CD8 co-receptor modifications to enhance T cell immunotherapy

A thesis submitted to the
Division of Infection and Immunity
of UCL
for the degree of
Doctor of Philosophy

By

Ignatius Chung Chua

Department of Immunology
Royal Free Hospital
Rowland Hill Street
London, UK

August 2013

Declaration

I, IGNATIUS CHUNG CHUA, confirm that this thesis is the result of my own work. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

TCR gene transfer can generate tumour antigen-specific T cells for adoptive immunotherapy. Following TCR gene transfer, transduced T cells usually display the same functional avidity as the parental clone from which the TCR was isolated. However, tumour-antigen specific T cells typically recognize over-expressed self-antigen and are often of low/moderate avidity. It is known that optimal recognition of target cells by CTL requires binding of the cognate peptide MHC class I complex (MHCI) by both TCR and the CD8 co-receptor. Some CD8β chain mutations have been shown to increase CD8 binding affinity with peptide/MHCI and enhance T cell effector function.

Murine CD8β chain mutants were generated affecting MHC binding sites (L58R, S53L, S54V and L58R/I25A) or glycosylation sites (T120A, T121A, T124A, and T120A/T121A/T124A). The mutated CD8β molecules were introduced into murine splenocytes using retroviral vectors together with tumour antigen-specific TCRs.

The CD8β mutants or control CD8β wild type (WT) chains were first introduced into CD8αα T cells obtained from CD8β knockout mice. All T cells were co-transduced to express the murine F5-TCR which recognizes the model tumour antigen, influenza A nucleoprotein (NP366) presented by H2-Db. The L58R MHC binding CD8 co-receptor mutant (L58R) demonstrated better IFN-γ and IL-2 production in response to relevant peptide while the CD8 glycosylation mutant (T120A/T121A/T124A) mutant demonstrated the opposite effect.

The in vitro function of CD4+ T cells transduced with F5-TCR showed that IL-2 and IFN-γ production was enhanced with CD8 co-receptor. In addition, introducing a L58R mutation in the CD8 co-receptor could further increase this effect. The effects of the human CD8 co-receptor with a homologous mutation (I59R) was also investigated in human CD4+ T-cells with a CMV-specific TCR.

In vivo studies showed that introducing the F5-TCR alone did not endow CD4+ T cells with significant protection against injected lymphoma cells expressing NP366. However adding CD8 co-receptor to the CD4+ T cells enhanced tumour protection. The genetically modified CD4+ T cells persisted for greater than three months in surviving mice and when re-challenged with antigen the CD4+ T cells with both F5-TCR and CD8 co-receptor had greater proliferative capacity and had more central memory phenotype cells.

Table of contents

List of figures and tables

Abbreviations

2D	two-dimensions
3D	three-dimensions
aa	amino-acid
Ag	antigen
AICD	antigen-induced cell death
APC	allophycocyanin
APC	antigen presenting cells
bp	base pairs
BCR	B cell receptor
BM	bone marrow
CMV	cytomegalovirus
conA	concanavalin A
CO2	carbon dioxide
cpm	counts per minute
^{51}Cr	^{51}chromium (sodium chromate)
Cα	constant domain of the TCR-α chain
Cβ	constant domain of the TCR-β chain
CDR	complementarity determining region
CTL	cytotoxic T lymphocyte
dH2O	distilled water
DN	double negative
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DLN	draining lymph nodes
DP	double positive
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
E:T	effector:target ratio

FACS	fluorescence-activated cell sorter
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FRET	fluorescent resonance energy transfer
GVL	graft-versus-leukaemia
GVHD	graft-versus-host disease
Gy	Gray
HSCT	haemopoietic stem cell transplantation
ICS	intracellular cytokine staining
IFN-	interferon-
IL-	interleukin-
IRES	internal ribosomal entry site
IS	immunological synapse
ITAM	immunoreceptor tyrosine based activation motif
kb	kilobases
Lck	p56lck tyrosine kinase
LCTE	low concentration tris-EDTA buffer
LFA-1	lymphocyte function-associated antigen-1
LN	lymph node
LTR	long terminal repeat
M	molar
m	milli (10^{-3})
μ	micro (10^{-6})
MDM2	murine double minute-2 protein
MFI	mean fluorescence intensity
MHCI	Major Histocompatibility Complex Class I
MHCII	Major Histocompatibility Complex Class I
MPSV	myeloproliferative sarcoma virus
MSCV	moloney sarcoma cell virus
mRNA	messenger ribonucleic acid

n	nano (10^{-9})
NDLN	non-draining lymph nodes
NK	natural killer
OD	optical density
p	pico (10^{-12})
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD1	programme cell death protein 1
PE	phycoerhthrin
PhAmpho	Phoenix-Amphotrophic packaging cells
PhEco	Phoenix-Ecotrophic packaging cells
PMA	phorbol myristate acetate
pMHCI	peptide loaded Major Histocompatibility Complex Class I
PTLD	post-transplant lympho-proliferative disease
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RT	room temperature
sc	subcutaneous
siRNA	small interfering ribonucleic acid
SP	single positive
SPL	spleen
SPR	surface plasma resonance
TAA	tumour associated antigen
TAP	transporter associated with antigen processing
Tcm	central memory T cells
Tem	effector memory T cells
TCR	T cell receptor
TCR-td	T cell receptor transduced
TGF-β	transforming growth factor-β

Th	T-helper CD4+ T cells
TIL	tumour infiltrating lymphocytes
TNF	tumour necrosis factor
Treg	T-regulatory CD4+ T cells
U	units
Vα	variable domain of TCR-α chain
Vβ	variable domain of TCR-β chain
WT	wild-type
WT1	Wilms tumour antigen 1
ZAP70	ζ-chain-associated protein

Chapter 1

Chapter 1. Introduction

This project investigates the potential of exploiting the CD8 co-receptor for T cell receptor (TCR) gene therapy of cancer. TCR gene therapy typically transfers TCR genes isolated from a single clone of antigen (Ag)-specific T cells. Retroviral transfer of cloned T cell receptor (TCR) genes has been shown to reliably re-direct the Ag specificity of T cells. This chapter provides background information on T cells, TCR structure, T cell signalling and the use of T cells for cancer immunotherapy.

1.1 Adaptive immunity: T vs B lymphocytes

The central function of the immune system is defence against infection. The adaptive immune system has evolved to distinguish small differences in molecular structure between foreign organisms (non-self) and native cells (self) through Ag-specific receptors on B and T lymphocytes (Nemazee 2000; Stritesky et al. 2012). The presence of additional danger signals from the innate system is often required for the adaptive system to target and eradicate pathogens (Banchereau & Steinman 1998; Pasare & Medzhitov 2005). Ag-specific receptors on B and T cells are generated by a process involving somatic gene recombination in the bone marrow and thymus. This allows the potential for a large number ($>10^{13}$) of Ag-specific receptors with different specificities (Nemazee 2000). The strength by which an Ag specific receptor binds to its ligand (antigen) is usually measured by its dissociation constant (K_d) and is known as receptor affinity. High affinity receptors (with low K_d) do not always have increased sensitivity to antigen (see Section 1.3). The antigen sensitivity of lymphocytes is often measured by detection of cytokines, proliferation or cytotoxicity in antigen titration experiments which is defined here as functional avidity. High avidity T cells are able to respond to low levels of antigen.

B cells and T cells are dissimilar in many aspects. B cell receptors (BCRs) bind to complex three-dimensional conformational determinants with high affinity, in contrast (Pierce & Liu 2010), T cell receptors (TCRs) bind short linear peptide fragments presented on major histocompatibility complex (MHC) molecules with lower affinity (Smith-Garvin et al. 2009). BCR secreted from B cells are called antibodies, which bind to surface Ag present on pathogens to trigger effector immune responses (Mikolajczyk et al. 2004; Corti & Lanzavecchia 2013). Unlike BCR, TCR are cell surface bound and target intracellularly processed peptide presented by MHC initiating T cell responses (Chaplin 2010). After encountering cognate Ag in the periphery, both B and T cells undergo expansion followed by a contraction phase (Krammer et al. 2007). Some cells may persist in the periphery as memory B or T cells (Mueller et al. 2012; Sanz et al. 2008). These cells which survive Ag-induced cell death (AICD) have augmented responses upon encountering the same Ag

(Williams et al. 2008). As the main focus of this thesis is T cells, there will be no further mention of B cells or BCR.

1.2 T lymphocyte development

Distinct stages in the development of thymocytes are marked by changes in cell surface molecules (Pear et al. 2004). Immature T lymphocytes, originating from the bone marrow, begin development in the thymus as double-negative (DN) T lymphocytes as there is no CD4 or CD8 expression. The DN3 thymocytes undergo gene rearrangement of the TCR-β chain and express an early form of TCR known as the pre-TCR (Fehling et al. 1995). These thymocytes later acquire both CD4 and CD8 expression and are known as double-positive (DP) thymocytes (Teh et al. 1988; Kisielow et al. 1988). The DP thymocytes undergo gene rearrangement for the TCR-α chain and express the complete TCR at low levels (Sebzda et al. 1999). DP thymocytes undergo positive selection in the cortex of the thymus following recognition of self-peptide/MHC and those that are not positively selected are deleted. During the process of positive selection DP thymocytes cease to express either the CD4 or the CD8 molecules and become single positive (SP) thymocytes. The role of CD4 and CD8 molecules in lineage selection is elaborated below in Section 1.4.2. The SP thymocytes migrate into the medulla and undergo negative selection (Morris & Allen 2012). During this process, T cells exhibiting strong interactions with self-Ag are deleted. One study showed that the negative selection for MHCI restricted TCR occur when the dissociation constant, K_d is <6μM (Naeher et al. 2007). During thymic development, only 2% of DP thymocytes survive the selection process and migrate into the periphery as SP CD4+ or CD8+ T lymphocytes. This process is responsible for central tolerance.

Peripheral T lymphocytes are identified by the presence of TCR and the CD3 complex (see Fig 1.1). The majority of T lymphocytes utilise the α- and β-TCR chains (as opposed to the γ- and δ-TCR chains) and as mentioned above express either the CD4 or the CD8 molecules. The TCR expressed by CD8+ T lymphocytes bind optimally to cognate peptide presented by MHC Class I (MHCI) and are described as MHCI restricted TCR, while the TCR expressed by CD4+ T cells bind to cognate peptide presented by MHC Class II (MHCII) and are likewise described as MHCII restricted TCR.

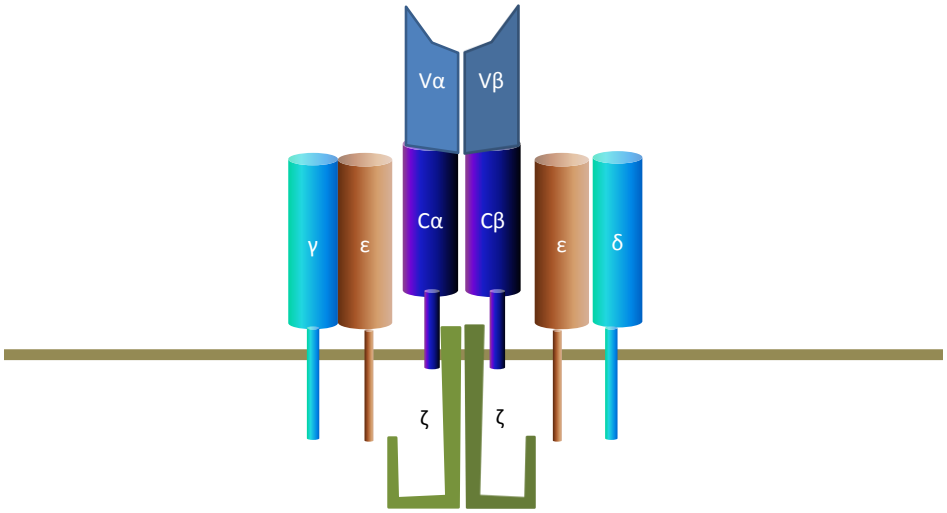


Figure 1-1 Schematic diagram showing components of the TCR/CD3 signalling complex.
TCR consists of variable (V)α or Vβ domains and constant (C)α or Cβ domains. The CD3 chains consist of ε, γ, δ, ζ which are closely associated with TCR. Immune tyrosine-based activating motifs (ITAMs) are found on the cytoplasmic tail of CD3 molecules which are below the cell membrane (grey line).

CD8+ and CD4+ T cells have different effector functions. CD8+ T cells mature into cytotoxic T cells (CTL) and release granzyme and perforin to directly kill target cells. CD8+ T cells can produce cytokines, mainly IFN-γ but also small amounts of IL-2 upon recognition of cognate Ag. Conversely, CD4+ T cells, also known as T-helper (Th) cells, are primarily cytokine producing cells. CD4+ T cells secrete a large number of cytokines to orchestrate the immune system. The sub-populations of mature CD4+ T cells are classified according to their cytokine signature: Th-1 cells produce IL-12, IFN-γ; Th-2 cells produce IL-4 and IL-5, T regulatory (Treg) cells produce TGF-β and IL-10; and Th17 cells produce IL-17, IL-21.

Both CD8+ and CD4+ T cells proliferate upon activation, following which a proportion of cells undergo AICD. The T cells surviving AICD can persist for long periods of time and become memory T cells. Memory phenotype T cells have the ability to respond rapidly on re-encounter with specific Ag without priming. CD8+ memory T cells are better characterised in the literature than CD4+ memory T cells (Marshall et al. 2011). Two distinct subtypes of memory exist; central memory T cells (Tcm) which express CD62L and CCR7 and home to lymph nodes and effector memory T cells (Tem), which circulate in the periphery without CD62L and CCR7 expression.

Of interest to this project is the influence CD4+ T cells have on the development of memory CD8+ T cells, particularly in the context of the non-infectious setting of cancer. The presence of Ag-specific CD4+ T cells, from the initial priming event, facilitates the induction and maintenance of memory CD8+ T cells and affects the quality of secondary responses. Research studies have shown that T cell help can occur either directly through an intermediary 'third cell' such as an Ag-presenting cell (APC) or through direct contact with the CD8+ T cell (de Goër de Herve et al. 2010). In many experimental settings, licensing through ligation of CD40 (on either APC or CD8+ T cell) by CD40L expressed on CD4+ T cells is thought to be a crucial factor. A second key mediator of T cell help is paracrine secretion of IL-2 from CD4+ T cells (de Goër de Herve et al. 2010), although a one recent paper suggests that autocrine IL-2 from the CD8+ T cell may be sufficient (Feau et al. 2011).

1.3 T cell receptor structure and signal transduction

The TCR propagates Ag-specific stimuli internally into the T cell thus initiating the effector response. The TCR is a hetero-dimer consisting of α- and β- chains linked by disulphide bonds (Fig 1-1). Each chain is formed of a variable region containing the Ag binding site and a constant region. The variable (V) regions, Vα and Vβ respectively, each contain sites known as complementary determining regions (CDRs) which are hyper-variable for amino-acid (aa) sequences and account for the large diversity of TCR specificities. The constant (C) regions have conserved aa sequences and function as a scaffold for the variable regions. The constant region contains trans-membrane and cytosolic domains which are important for transduction of TCR signalling to downstream molecules.

Following thymic deletion of T cells expressing TCR with high affinity to endogenous-peptide/MHC, most peripheral T cells express TCR of low to moderate affinity. Cognate peptide may be derived from endogenous (self) or proteins from a foreign source e.g. pathogens (non-self). Low affinity interaction with self-peptide/MHC is required for survival of naive T cells in the periphery and was shown to enhance recognition of foreign antigen especially when the foreign-peptide (agonistic)/MHC surface density is low (Irvine et al. 2002; Krogsgaard et al. 2005). An explanation for this process is that self-peptide/MHC can form pseudo-dimers with agonistic-peptide/MHC resulting in aggregates and clustering (Krogsgaard et al. 2007).

Structural studies show that the Ag-binding site of the TCR is orientated diagonally to the long axis of the peptide/MHC (pMHC)-binding groove (Garcia et al. 1996; Garboczi et al. 1996). The CDR1 and CDR2 regions of the TCR form conserved contacts with MHC while the CDR3 region forms contacts with aa residues of the presented peptide situated in the MHC groove (Rudolph et al. 2006). As TCR have to screen a large number of pMHC complexes, it is postulated that a two-step process may occur where

CDR1 and CDR2 initiate contact with MHC and then allow CDR3 to 'sample' the peptide groove (Wu et al. 2002). The angle of the diagonal is variable for different TCRs and may determine the CD8 dependency of the TCR; four TCRs with Vα chains that approximated with the N-terminal end of the bound peptide were found to be CD8 dependent whereas in a different four TCRs where the Vα chain approximated to the C-terminal end of the bound peptide were found to be CD8 independent (Buslepp et al. 2003). Structural studies of the TCR-pMHC interaction suggest that most of the TCR binding energy is focused on the peptide which allows discrimination between self and non-self (Lee et al. 2000; Simpson et al. 2011). The role of co-receptors in this process is discussed separately below (Section 1.4).

As introduced in Section 1.1, the strength or degree by which TCR binds to pMHC is known as TCR affinity (k_d). The affinity of most naturally selected TCR, measured using surface plasmon resonance (SPR), are typically between 1-100 μM. These are relatively low affinity interactions compared to antibody binding affinities (pM-nM range). To measure TCR affinity using SPR, MHC molecules coated on a metallic surface are allowed to interact with monoclonal TCRs in a liquid solution. When interaction between TCR and MHC occurs the aggregation of TCR/MHC complexes increase the surface 'roughness' and interrupts an electromagnetic wave propagating on the metal surface (surface plasmons) causing light to be emitted and detected with a photo-detector. Recently, a new method using red blood cell coated with MHC is thought to be more physiologically relevant as the molecules of interest are on a membrane which allows two-dimensional (2D)-interaction to be measured. The experimental data using the 2D method suggested that TCR first binds to MHC followed by enhancement of binding by CD8 co-receptor in a two-stage process. Within the TCR-α constant membrane proximal region is a conserved motif known as the connecting peptide motif (CPM) which when mutated reduces CD3δ association (Bäckström et al. 1998) and diminishes antigen responsiveness (Bäckström et al. 1996). The reason for CPM deficient TCR inability to utilize CD8 co-receptor (Naeher et al. 2002) was attributed to the failure of co-receptor approximation (Mallaun et al. 2008).

In order for the TCR to be expressed on the cell surface it has to form a stable heteromeric complex with the CD3 molecules (γ, δ, ε, ζ) on the T cell membrane (Fig 1-1). The different CD3 chains contain immune tyrosine-based activating motifs (ITAMs) in the cytoplasmic domain which when phosphorylated recruit downstream signalling molecules. The exact molecular structure for the TCR/CD3 complex has not yet been elucidated but molecular studies have revealed that CD3 molecules are heterodimers with the CD3ε chain pairing with either CD3δ (Sun et al. 2004; Arnett et al. 2004) or CD3γ (Sun et al. 2001; Kjer-Nielsen et al. 2004). Recent studies have suggested that the CD3δ and γ chains associate with the TCRα and β chains

respectively (Kuhns et al. 2010; Kim et al. 2010; Kuhns & Davis 2007). The CD3ε chains contain a proline-rich sequence, which undergoes conformational change after TCR ligation, and is involved in the recruitment of adaptor Nck (Gil et al. 2002; Gil et al. 2005). The importance of this region for TCR signalling is unclear as Nck was shown to be dispensable for T cell development (Szymczak et al. 2005) and its role may be limited to low avidity TCR interactions (Tailor et al. 2008) or related to regulating TCR/CD3 expression (Mingueneau et al. 2008; Wang et al. 2010). The CD3ζ chains, unlike the other CD3 molecules, are mostly cytosolic and form homodimers with six ITAM motifs (Call et al. 2002). The ITAMS of CD3ζ may be buried in the negatively charged lipid membrane as a result of basic residue rich domains (Aivazian & Stern 2000) and released when TCR conformational change occurs (Gagnon et al. 2010). Currently, there is still uncertainty of how TCR ligation induces conformational change within the TCR/CD3 complex resulting in phosphorylation of ITAMs by serine kinases Lck or Fyn.

The aggregation of TCRs at the T cell/APC interface due to presence of MHC complexes may trigger T cell signalling and increase the concentration of signalling complexes. The exclusion of large signalling inhibitive molecules such as CD45 phosphatases and the aggregation of pro-signalling molecules such as TCR, CD3, Lck has been observed by confocal microscopy. This ordered structure is known as the immunological synapse (IS). The mature IS consists of distinct zones with the TCR/CD3 clustered in the centre and adhesion molecules such as LFA-1 in the periphery. However, as the IS can take up to 30 minutes to form, it was argued by some that TCR triggering does not require the formation of the IS and that the IS has a regulatory role in TCR signalling (Lee et al. 2002). Subsequently the initiation of TCR triggering was found to occur on microclusters of TCR/CD3, which form within seconds of T cell/APC interaction (Bunnell et al. 2002; Yokosuka et al. 2005). Using advance microscopy techniques, Varma et al (Varma et al. 2006) discovered that TCR/CD3 microclusters have as little as 11-17 TCR molecules. Several microclusters may coalesce to form larger clusters before migrating towards the central zone of the IS where they are degraded. The microclusters do not contain CD45 phophatases suggesting that tyrosine kinases within the microcluster are in an active state. Inhibition of microclusters using MHC blocking antibodies abrogates formation of new microclusters and T cell calcium flux.

Recent studies of real time in-vivo data of T cell/APC interaction have shown that high affinity TCR/pMHC interactions lead to the formation of stable synapses and strong TCR signalling, whereas low affinity TCR/pMHC interactions lead to short-lived synapses and weaker TCR signalling (Henrickson et al. 2008; Moreau et al. 2012). Related to this, another group found that only high affinity interactions result in a prolonged expansion phase (Zehn et al. 2009).

Down-stream signalling after phosphorylation of ITAMs is complex and involves a multitude of tyrosine kinases, adaptor molecules, secondary messengers and transcription factors. As this is beyond the scope of this thesis, the intricacies are not discussed here. Three divergent downstream pathways lead to activation of transcription factors NFAT (calcium flux), NF-κB and AP1 which result in proliferation, production of cytokines, cytoskeletal changes and degranulation.

1.4 The CD8 co-receptor

The CD8 and CD4 co-receptors are surface markers for MHCI and MHCII restricted T cells respectively. Both co-receptors play an important role facilitating proximal TCR signalling and have elongated glycoprotein structures allowing engagement with their respective MHC molecules. The cytoplasmic tails of both receptors contain motifs which allow binding of Lck a crucial tyrosine kinase involved in TCR proximal signalling. As the thesis relates to the use of a MHCI restricted TCR and the use of mutated CD8 co-receptor molecules, this section will focus on the CD8 co-receptor.

1.4.1 CD8 co-receptor: from structure to function

1.4.1.1 Comparisons between two naturally existing forms of CD8 co-receptor

The CD8 co-receptor is a glycoprotein dimer with each dimer containing an immunoglobulin like domain (Fig 1-2). The most prevalent CD8 co-receptor on thymocytes and conventional T cells is in the form of a heterodimer consisting of α- and β- chains. The CD8 co-receptor can also exist as α-chain homodimer in a wide range of immune cells including T, natural killer and dendritic cells. Despite the large differences between the sequence of the CD8α and CD8β chains, predictions based on molecular structure suggest that both have a similar topology which is confirmed when the crystal structure of CD8αβ co-receptor is finally made available (Wang et al. 2009).

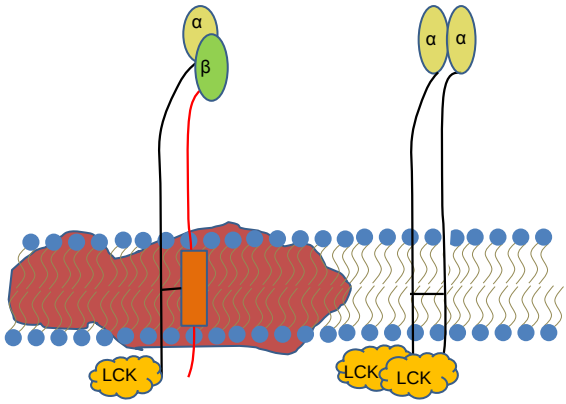


Figure 1-2 CD8 co-receptor can exist in two forms.
The CD8 co-receptor on conventional T cells is a heterodimer (left) consisting of immunoglobulin like α (light green) and β chain (green), whereas it can also exist as a CD8αα homodimer (right) in T and non-T cells. The α chain contain a motif which allow engagement with LCK (yellow) The β chain is palmitoylated (orange), which allows partition onto the lipid raft which was thought to improve TCR signalling.

Affinity studies have shown that CD8αα and CD8αβ have similar binding strengths to MHCI molecules (Sun & Kavathas 1997; Kern et al. 1999). Even though the CD8αα has been shown to be able to induce proximal signalling in hybridomas, it is generally acknowledged that CD8αβ is the functional homologue of the TCR signalling apparatus (Gangadharan & Cheroutre 2004). However without the CD8β chain, most CD8+ T cells do not survive thymic selection and CD8β-/- mice have few peripheral CD8+ T cells (Fung-Leung et al. 1994; Crooks & Littman 1994). When tested the CD8β-/- CD8 T cells have decreased sensitivity to antigen in functional assays (Witte et al. 1999; Bosselut et al. 2000; Potter et al. 2001; Arcaro et al. 2001). Although one study showed that the CD8αα homodimer does not co-localise with TCR (Cawthon & Alexander-Miller 2002), a recent fluorescent resonance energy transfer (FRET) based imaging study performed by Rybakin showed there was no difference in recruitment of either forms of CD8 to the immunological synapse (Rybakin et al. 2011).

The CD8αα molecule has a higher affinity (k_d=10μM) for the non-classical MHC molecule thymic leukaemia antigen (TL antigen) than MHCI (Liu et al. 2003). TL antigen expression on intestinal epithelial was shown to mediate cell death of CD8αβ+ T cells that do not express CD8αα molecule. It was discovered that strong T cell activation up-regulates the CD8αα homodimer which sequesters TL away from CD8αβ and protects these cells from FAS induced cell death. Such a mechanism may result in affinity maturation within the gut, as surviving T cells are typically high avidity memory T cells (Huang et al. 2011).

1.4.1.2 Structural and molecular aspects of the CD8 co-receptor

The CD8 co-receptor is a transmembrane glycoprotein consisting of two immunoglobulin-like domains suspended from the cell membrane by an elongated stalk, which extends through the cell membrane into the cytoplasm (Fig 1-3). The apex of the CD8 co-receptor consisting of two globulin domains associates with the conserved regions of MHC-I through non-convalent bonds in a bidentate manner. The earlier structural studies of the CD8 co-receptor using crystallography of CD8αα co-receptor ligating with TL antigen or human and murine MHC molecules (Gao et al. 1997; Kern et al. 1998; Liu et al. 2003) showed that the globular domains are situated on either side of the α3-domain, which is distant from the TCR binding site (Connolly et al. 1990), in a clamp like fashion.

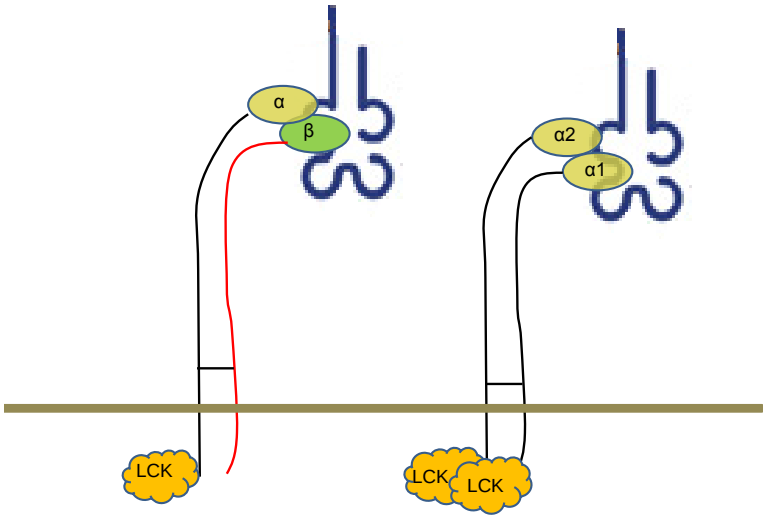


Figure 1-3 CD8 co-receptor engages with the α-3 domain of MHCI The CD8 heterodimer (left) and CD8 homodimer (right) have similar elongated stalks with immunoglobulin domain in the distal most portion which engages the α3 domain of MHCI (shown in blue outline).

The long axis of the globular domains is perpendicular to the long axis of the MHCI molecule (Fig 1-3) with slight asymmetry: one globular domain in the T cell proximal position is assigned as CD8α1 and the other domain in the T cell distal position is assigned as CD8α2. The CD8α1 globulin domain accounts for about 70% of the binding site and also makes some contact with the MHC-I α2 and β2-microglobulin domains. There have been conflicting predictions on whether the CD8β chain in the CD8αβ molecule occupies the upper CD8α1 or lower CD8α2 positions: predictions based on stalk length suggest the former (Kern et al. 1999), predictions based on surface electrostatic charge (Devine et al. 1999) suggest the latter and extrapolations based on mutagenesis data suggest that both positions are possible (Chang et al. 2006; Devine et al. 2006). This uncertainty was only resolved when the crystal structure of the CD8αβ molecule with H2-Dd-MHC molecule was finally solved showing that CD8β occupies the T cell proximal CD8α1 position and only contacts the

α3-domain of the MHC-I (Fig 1-4), contributing about 50% of the total binding energy (Wang et al. 2009).

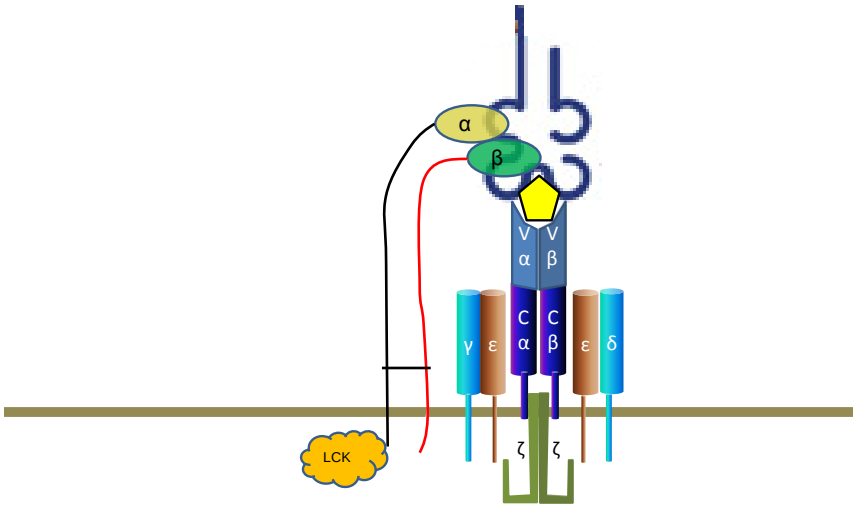


Figure 1-4 The TCR and CD8 co-receptor engage pMHCI at two distinct points.
When cognate interaction between TCR/CD3 complex (central structure annotated in Fig 1-1) and pMHCI (yellow pentagon with blue outline above) occurs, the CD8 coreceptor (elongated heterodimer on the left of TCR) also engages MHCI and becomes closely associated with TCR/CD3 complex. As the LCK is attached to the tail of CD8α chain, this also engages the ITAMs in the cytoplasmic portion of the CD3 chains forming a second point of contact.

The CD8α and CD8β stalk regions are rich in proline, serine and threonine residues and both contain N and O-glycosylation sites which allow post-translational modification. The chemistry of the stalks allow an extended rigid conformation but with some flexibility (Fig 1-2 and 1-3). The CD8β stalk is shorter than the CD8α stalk and contains one N-glycosylation and three O-glycosylation sites which are highly conserved between different species. Removing the CD8β stalk and replacing it with the CD8α stalk results in failure of CD8+ SP T cell selection in the thymus (Rettig et al. 2009). Performing the reverse and removing the CD8α stalk and replacing it with the CD8β stalk improves the sensitivity of T cells to antigen (Wong et al. 2003). These results emphasise the important contribution of the CD8β chain to the function of the CD8 co-receptor (Renard et al. 1996). The high level of conservation of the CD8β stalk region between different species adds further weight to this interpretation. The role that the CD8β plays in optimizing TCR signalling was thought to be related to the intramembrane portion of CD8β containing sequences for palmitoylation which is regarded as important for lipid raft partitioning. However, this has been disputed because CD8β with mutated palmitoylation sequences were still functional and CD8α also contains sequences for palmitoylation (Pascale et al. 1992; Fragoso et al. 2003). As mentioned above, the CD8β stalk region is essential to the

co-receptor function and was shown to enable CD8 association with CD3δ and the CPM motif of the TCR-α chain (Wheeler et al. 1998; Doucey et al. 2003; Mallaun et al. 2008; Naeher et al. 2002). It is postulated that the CD8β stalk forms the centre portion of a TCR co-receptor zipper model and allows optimal orientation with the TCR/CD3 complex to facilitate signalling (Palmer & Naeher 2009) (Fig 1-5).

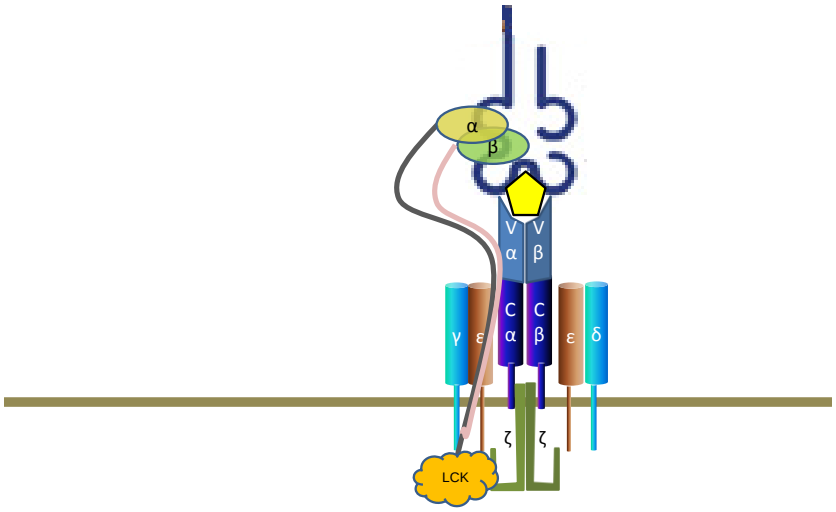


Figure 1-5 The CD8 co-receptor zipper model.
Naher and Palmer proposed that the CD8 co-receptor forms a tight association, like a zip, with the TCR after antigen ligation. Using the TCR/pMHCI/CD8 structure shown in Fig 1.4 to illustrate this, a further third point of engagement is postulated to occur between the CD8β stalk (pink line) with the CPM motif within the Cα (dark blue) chain of TCR.

Lastly, the cytoplasmic portion of the CD8α chain contains a CxC motif that allows association with Lck (Zamoyska et al. 1989; Turner et al. 1990). This is similar to the CD4 co-receptor although the CD4 co-receptor contains two extra CxC motifs, which potentially allow for increased Lck binding (Wiest et al. 1993; Shaw et al. 1989; Erman et al. 2006).

1.4.1.3 Role of CD8 co-receptor in TCR proximal signalling

The close physical association of the CD8 co-receptor and TCR on lipid rafts has been well documented in many studies (Takada & Engleman 1987; Beyers et al. 1992; Suzuki et al. 1992; Gallagher et al. 1989). Structural studies have also shown that in the tri-molecular assembly of TCR/pMHCI/CD8 the TCR and CD8 engage the same pMHCI molecule at distinct positions. Therefore, it is envisaged that biologically, TCR and CD8 on the T cell (in *cis*) will interact with pMHC on an APC (in *trans*). However, as the TCR and CD8 co-receptor have different binding affinities to the pMHCI (Wyer et al. 1999), simultaneous engagement is unlikely. Evidence that the CD8 co-receptor is the first to engage MHCI was based on experiments using fluorescence

correlation spectroscopy of T cells stained with multimer. It was found that the early association rates (κ_{on}) were not related to pMHCI structure and could be blocked using anti-CD8 antibodies (Gakamsky et al. 2005). Furthermore, the early association rates determined in this study were similar to the association rate of CD8 co-receptor and MHCI using SPR (Wyer et al. 1999) but not between TCR and MHC (Gakamsky et al. 2004). The authors suggested that CD8 co-receptor enhanced the TCR-pMHCI interaction by increasing the number of TCR/MHC complexes (Pecht & Gakamsky 2005). Others have postulated that the fast CD8 co-receptor engagement can re-orientate MHC from a supine position (Mitra et al. 2004) into a more convenient position for TCR engagement.

Other studies have suggested that the TCR first engages MHCI, in keeping with the function of TCR as an Ag-specific receptor triggering positive signalling only when cognate pMHCI is encountered (Irvine et al. 2002). Zal and Gascoigne found that the FRET between CD8 co-receptor and TCR only occurred during cognate interactions. Experiments using RBC coated with pMHCI molecules to measure the TCR and CD8 interaction, demonstrated a biphasic association with the early phase involving the TCR and the second phase involving the CD8 co-receptor (Jiang et al. 2011). Consequently, current opinion favours the hypothesis that TCR binds to pMHCI first followed by recruitment of CD8 co-receptor and Lck by association (van der Merwe & Dushek 2011; Laugel et al. 2011; Edwards et al. 2012). Surprisingly, the cooperative effects of CD8 co-receptor in cell-cell adhesion requires phosphorylation of ITAMs by free Lck (Jiang et al. 2011). The role of free Lck in proximal signalling was first mentioned in a study examining alloreactive and anti-viral T cell responses in CD8 and CD4 co-receptor deficient mice (Schilham et al. 1993). The same mechanism may explain how high affinity MHCI restricted TCR generated through affinity maturation can function in CD4+ T cells (Robbins et al. 2008). However, in the context of weak TCR/pMHC interactions, TCR ligation may still allow recruitment of free Lck to partially phosphorylate CD3 associated ITAMs. Jiang et al showed that the initial step involving free Lck is required for cooperative effects mediated by the CD8 co-receptor. It has been postulated that CD8 co-receptor recruitment to the TCR activation site depends on intracellular attributes such as complementarity between the much stronger Lck/LAT interaction (Gibbings & Befus 2009); this is known as the 'inverted model' as it is independent of MHC binding by the extracellular portion of CD8 co-receptor. Experimetal data from a number of groups supports this model (Xu & Littman 1993; Thome et al. 1996; Purbhoo et al. 2004).

There can be two possible explanations for the cooperative effect of CD8 co-receptor on TCR function; firstly it enhances stabilization of TCR/pMHCI interactions (Luescher et al. 1995; Garcia, Scott, et al. 1996; Cebecauer et al. 2005) and secondly it facilitates transport of Lck to the vicinity of the TCR signalling complex. The

importance of the first effect has been disputed as CD8 co-receptor affinity for MHCI is several logs lower than the affinity of TCR for pMHCI, CD8 aggregation is independent of MHC molecules (Wooldridge et al. 2003; Buslepp, Kerry, et al. 2003) and site-directed mutagenesis of MHCI reducing affinity of TCR binding have been shown to have little effect on T cell signalling. However mathematical modelling suggests that the extracellular portion of the CD8 molecule increase ligand interaction 30-fold with respect to recruitment of Lck (van den Berg et al. 2007) rather than stabilisation of TCR/pMHCI (Artyomov et al. 2010).

The transportation of Lck to the TCR signalling complex is critical for CD8 co-receptor function. It has been demonstrated that Lck mutagenesis abrogates recruitment of CD8 (Xu & Littman 1993) and mutagenesis of the Lck binding site on the CD8 co-receptor impairs T cell activation (Arcaro et al. 2001). CD8-associated LCK optimizes the phosphorylation of ITAMS allowing TCR signal transduction (Purbhoo et al. 2001). The palmitoylation of the CD8β chain facilitates partitioning of the CD8 with the TCR on the same lipid raft thus enhancing this effect.

1.4.2 Role of the CD8 co-receptor in the thymus

Immature CD8+ CD4+ DP thymocytes have to be able to recognise MHC molecules and discriminate them from non-MHC molecules in order to undergo positive and negative selection. During this early stage in T cell development, Lck levels are low and are mostly co-receptor associated (Wiest et al. 1996). This restricted distribution of Lck is instrumental in imposing MHC specificity as the remaining Lck is sequestered by CD8 or CD4 co-receptor. Murine immature thymocytes deficient in CD8 and CD4 co-receptors have higher levels of free Lck and can be activated more easily with anti-TCR antibodies compared to wild-type immature thymocytes. In an MHC-deficient environment, these thymocytes can mature and cause autoimmune disease in the periphery via the targeting of non-MHC targets in vitro (Park et al. 2007).

TCR triggering requirements of positive and negative selection are different; for positive selection a high number of low affinity interactions are necessary (Hogquist et al. 1994) whilst during negative selection a low number of high affinity interactions are sufficient (Ebert et al. 2008; Alam et al. 1996; Williams et al. 1999). The CD8 co-receptor has been shown to play a role in modulating the strength of TCR signalling through post-translational glycosylation during T cell development (Daniels et al. 2001; Baum 2002; Gascoigne 2002). Immature DP thymocytes were found to bind non-cognate MHCI multimers better than mature CD8+ SP thymocytes (Daniels et al. 2001). These changes occurred through the addition or removal of sialic acid moieties on O-linked glycans on conserved threonine residues on the CD8β chain under the influence of ST3-Gal-1 enzyme (Moody et al. 2001). On immature

thymocytes the CD8 co-receptor was found to exhibit lower levels of cell surface sialylation. *Ex vivo* mature thymocytes when desialylated artificially by neuraminidase were found to bind more strongly to MHC multimers, suggesting that desialylated CD8 co-receptor could improve TCR signalling and increase positive selection (Daniels et al. 2001).

After positive selection in the thymus, it is possible that the sialylation of O-linked glycans of the CD8 co-receptor may help mature thymocytes avoid negative selection. Subsequently it was found that the post-translational sialylation changes occur on the three threonine residues (T120, T121 and T124) that are conserved between all species studied to date (Moody et al. 2003). It was suggested that the presence of sialic acid in the stalk region gives rise to electrostatic repulsion effects between CD8 and pMHCI (Moody et al. 2003; Rudd et al. 1999), however thermodynamic considerations make this mechanism unlikely (Shore et al. 2005). A more plausible explanation is that the O-glycans, which are on the membrane distal region of the CD8 stalk, may influence the stalk conformation such that it reorientates the CD8 immunoglobulin domain and affects engagement with the MHCI molecule (Shore et al. 2005).

One of the significant stages of thymocyte development is lineage selection. This lineage divergence is largely determined by the MHC restriction of the TCR (Singer et al. 2008); TCR that have greater affinity for MHCI lose CD4 expression and become CD8+ SP T cells, TCR that have greater affinity for MHCII lose CD8 expression and become CD4+ SP T cells. The process involves an intermediate stage where upon engagement of TCR by MHC thymocytes down-regulate CD8 and become CD4+ CD8lo thymocytes (Brugnera et al. 2000). The CD8 downregulation was also observed with T cells are engaged only by MHCI (Bosselut et al. 2003). Therefore downregulation of CD8 gene transcription is considered to be a reliable indicator of the intermediate stage phenotype between DP and SP thymocytes (Singer 2002). During CD8 co-receptor downregulation, the persistence of a positive signal results in CD4+ differentiation (Liu & Bosselut 2004). In the opposite situation when a positive signal diminishes, the cell receives survival signals from IL-7 in a process known as 'co-receptor reversal' where CD4 gene transcription is silenced and CD8 gene transcription is re-initiated (Yu et al. 2003) through the activation of E8I enhancer elements (Park et al. 2007). Silencing of the CD4 gene in experimental conditions also results in intermediate thymocytes differentiating into CD8+ T cells (Sarafova et al. 2005). Intermediate thymocyte CD8 and CD4 genes are regulated in a co-ordinated fashion by the transcription factors Th-POK (T-helper-inducingPOX/Kruppel factor) and Runx, which have reciprocal actions on CD8 and CD4 genes. Th-POK induces CD4 expression and silences CD8 gene expression (He et al. 2005; Sun et al. 2005), Runx silences CD4 expression by binding to Th-POK while promoting CD8

enhancers (Taniuchi et al. 2002). Recently, it was found that Runx mediates the physical association of CD8 and CD4 genes, which are separated by 53.3 megabases, to enable coordinate gene regulation (Collins et al. 2011).

1.4.3 Role of the CD8 co-receptor in peripheral T cells

The CD8 co-receptor is important for tuning TCR responses in peripheral CD8+ T cells. In the periphery, expression levels of the CD8 co-receptor are lower than in thymocytes and modulated through gene transcription (Zamoyska & Parnes 1988). Even before Ag encounter, naïve CD8+ T cells require interaction with MHCI molecules for survival and may be able to up-regulate CD8 co-receptor expression in order to enhance sensitivity to low affinity antigens (Takada & Jameson 2009). In vitro experiments which show that the CD8 co-receptor contributes significantly to the function of peripheral MHCI restricted T cells (Holler & Kranz 2003). In the rare event that a strong interaction with self-Ag occurs, the CD8 co-receptor can be down regulated to prevent autoimmunity (Teh et al. 1989; Zhang et al. 1995). As mentioned in Section 1.3, self-Ag can enhance low density agonist-pMHCI interaction and this was shown in a viral Ag model to be a CD8 dependent process (Anikeeva et al. 2006).

Modulation of CD8 expression can be influenced by γ-chain cytokines (IL-2, IL-4, IL-7 and IL-15) through the JAK-STAT pathway, this was found to be independent of cell metabolism which is driven by phosphatidylinositol-3OH kinase (Park et al. 2007). It is thought that during homeostatic conditions, weak interactions between TCR and self-pMHC allow IL-7 to mediate γ-chain signalling through IL-7R to up-regulate CD8 expression. This dynamic feedback loop is known as 'CD8 tuning'. However, there are other studies that have shown that IL-2 and IL-4 exposure results in downregulation of CD8 expression (Erard et al. 1993; Kienzle et al. 2002; Maile et al. 2005; Kambayashi et al. 2001). The interaction between cytokines and CD8 expression can be complex, for example the effects of IL-4 mediated down-regulation of the CD8 co-receptor can be ameliorated by the presence of IFN-γ (Apte et al. 2008). CD8 downregulation has been seen in chronic infections including human immune-deficiency virus infection (Schmitz et al. 1998), *Trypanosoma cruzi* infection in mice (Grisotto et al. 2001) and Echinococcus locularis infection in mice (Kizaki et al. 1991). During acute infections to *Listeria monocytogenes* and Vaccinia virus there is transient downregulation of the CD8 co-receptor, mediated by IFN-γ, which results in loss of pMHCI binding and reduction of T cell response to antigen (Xiao et al. 2007). Downregulation of CD8 co-receptor occurs between four to eight days after Lymphocytic choriomeningitis infection (LCMV) (Slifka & Whitton 2000). However, the Ag experienced memory T cells have ten-fold higher functional responses despite CD8 co-receptor down-regulation, which may be related to higher levels of Lck (Slifka

& Whitton 2001). However, Jameson et al demonstrated that the Ag experienced CD8+ T cells did *not* have increased functional responses and remained dependent on the CD8 co-receptor for antigen recognition. This discrepancy may be related to the different MHCI alleles used by Jameson (Kb) vs Slifka (Db) (Xiao et al. 2007).

Desialylation of the O-linked sugars of the CD8β chain occurred after cognate antigen recognition following the vaccination of F5-TCR transgenic mice with relevant peptide (Casabó et al. 1994). Although there is no direct evidence that desialylation of CD8β enhances T cell function in the periphery, two studies using neuraminidase enzyme to reduce total cell surface sialic acid on naïve and activated T cells resulted in enhanced T cell function (Pappu & Shrikant 2004; Kao et al. 2005).

The CD8 co-receptor is part of a negative feedback signalling network that modulates responses within a narrow physiological range even when there are large differences in signalling molecule expression (Feinerman et al. 2008). Recently the fine-tuning effects of CD8 co-receptor were also demonstrated for TCR interaction with endogenous peptides; using a panel of self-peptides with agonistic or antagonistic properties, presence or absence of CD8 co-receptor had different effects on the T cell responses. For example, an antagonistic peptide in the absence of CD8 co-receptor became a co-agonist in CD8+ T cells (Stone et al. 2011). It is possible that the CD8 co-receptor allows the T cell to have active control over which antigen to 'focus on' to counter the effects of TCR degeneracy thus discriminating recognition of self or non-self (Laugel et al. 2011).

1.5 Immune surveillance and the immune response to tumours

It was postulated as early as the 1900s that the immune system has an important role in preventing cancer in long-lived organisms (Ehrlich 1909). The evidence for the theory of immune surveillance is in part the increase incidence of cancers observed in immune-compromised individuals (Vajdic & van Leeuwen 2009; Chua et al. 2008) together with the association of tumour infiltrating CD8+ T cells and NK cells with better prognosis (Hayakawa et al. 2011; Nelson 2008). The early research that led to the breakthrough findings that immune cells can detect tumour associated antigen (TAA) and differentiate cancer cells from normal cells (Old & Boyse 1964) have provided the basis for Burnet to propose the cancer immune surveillance hypothesis, which predicted that the immune cells were responsible for inhibiting cancer growth in healthy hosts. This can occur through the elimination of viral infections which can induce tumours; the prevention of a tumourigenic inflammatory environment; and the elimination of spontaneous tumours expressing ligands for activating receptors of innate and adaptive immunity (Schreiber et al. 2011).

The identification and characterisation of tumour antigens followed the use of carcinogens, viruses or ultraviolet irradiation to induce tumour in animal models. The

tumour antigens were found to be products of mutated cellular genes, abnormally expressed normal genes or non-human viral genes. Human tumour antigens can be divided into two main categories- tumour specific antigens (TSA) and tumour associated antigens (TAA). Tumour specific antigens include differentiation antigens (melanoma), mutated oncogenes (p53, RAS), viral antigens (human papilloma virus proteins) and germline antigens (NY-ESO-1) whilst tumour associated antigens are typically overexpressed cellular antigens (Wilms tumour antigen-1 and murine double-minute [MDM] antigen), which are also expressed at lower levels in normal tissue. TSA are often presented poorly on the cell surface due to competition with the large number of peptides derived from normal cellular proteins for MHC and may not be amenable for immune targeting.

Animal models have allowed the immune elements important for cancer surveillance to be dissected; cellular elements including CD8+ cytotoxic T lymphocytes (CTLs), CD4+ Th1 helper T cells and natural killer (NK) cells were crucial in limiting the formation of carcinogen induced tumours (Teng et al. 2008; Kim et al. 2007). Animals without T and NK cells such as RAG2-/- mice had an even more profound susceptibility to tumour (Kaplan et al. 1998; Shankaran et al. 2001). Among the cellular products, the cytokine IFN-γ was especially important in mediating rejection of transplanted tumour cells (Dighe et al. 1994). Immuno-editing is a more recent model describing the dynamic process between the immune system and the immunogenic phenotype of tumours (Schreiber et al. 2011). In this model, immunogenic cancer cells are eliminated but less immunogenic cancer cells are left behind with the potential to escape. The less immunogenic cancer cells do not immediately proliferate to generate large tumours but may go through an equilibrium phase where outgrowth is inhibited by the immune system. This is also known as the dormant phase where latent tumour cells may lie quiescent for years before progression (Aguirre-Ghiso 2007). Both innate and adaptive immunity are involved in the elimination phase via type 1 interferon which activate dendritic cells and promote induction of adaptive anti-tumour responses (Schreiber et al. 2011). An elegant study showed that adaptive immunity involving CD8+ and CD4+ T cells and the cytokines IL-12 and IFN-γ were accountable for tumour latency (Koebel et al. 2007).

Others have long supported a mouse model of spontaneous tumour development and have suggested instead that tumour escape occurs because of induction of tumour tolerance rather than loss of intrinsic immunogenicity (Willimsky & Blankenstein 2005). This process was found to occur at a premalignant stage and is associated with non-responsive CD8+ T cells. Hence although tumour immunogenicity exists, the elicited immune response is non-destructive (Willimsky et al. 2008). To reconcile these alternate models, Pradeu and Carosella suggested that tumour immunogenicity does not only depend on the presence of a sufficient density of novel antigens but

that these antigens have to undergo repeated modifications (Pradeu & Carosella 2006). This concept has been appropriately paraphrased by Blankenstein as 'antigen discontinuum' (Blankenstein et al. 2012).

There are several immune-modulatory mechanisms by which tumour cells escape immune-surveillance. The most common cause is MHCI down-regulation, which frequently occurs in human tumours (Garrido et al. 2010). Defects in antigen processing also cause a similar form of resistance (Dunn et al. 2002). Prominent tumour associated immunosuppressive effects include expression of programme cell death protein 1 (PD1) ligands; production of indoleamine-pyrrole-2, 3-dioxygenase (IDO) (Uyttenhove et al. 2003) or tryptophan-2,3 dioxygenase (TDO) which depletes tryptophan (Opitz et al. 2011; Pilotte et al. 2012); production of galectin-3 which reversibly impairs T cell activation (Demotte et al. 2010); and lastly production of lactic acid, prostaglandins and transforming growth factor β (TGF-β). The tumour milieu is not only low in oxygen and difficult to penetrate (Trédan et al. 2007), but there are non-tumour cells, which have direct immunosuppressive effects including Tregs (Curiel et al. 2004; Getnet et al. 2009) and myeloid suppressive cells (Gabrilovich & Nagaraj 2009). Here, tumour infiltrating lymphocytes (TILs) have an 'exhausted' phenotype with upregulation of T cell immunoglobulin and mucin domain-containing protein 3 (TIM3), lymphocyte activating gene 3 (LAG3), PD1 and cytotoxic T lymphocyte antigen 4 (CTLA-4) (Baitsch et al. 2011). The information gleaned from understanding tumour associated immune-modulatory mechanisms has led to the identification of novel approaches to target cancer through pharmacological agents, monoclonal-antibodies and reprogramming of T cells (Blankenstein et al. 2012).

1.5.1 The role of CD8+ T cells in tumour immunity

One of the major hallmarks of tumour cells is uncontrolled growth. Many of the unique tumour antigens are derived from intracellular proteins that are not expressed on the tumour cell surface but need to be processed internally into peptide fragments, loaded onto MHCI and presented on the surface. As MHCI is present on almost every nucleated cells, tumour specific MHCI restricted T cells are therefore theoretically able to directly engage and kill most tumours. Not surprisingly many published reports have focused on utilizing CD8+ T cells for cancer immunotherapy (Kast et al. 1989; Riddell & Greenberg 1995; Yee et al. 2000) including more recent publications on adoptive transfer of ex vivo expanded tumour reactive CD8+ T cells with response rates of up to 70% (Rosenberg et al. 2011). The principle mechanism of CD8+ cytotoxic T lymphocyte (CTL) killing is the release of specialized lytic granules containing perforin and granzymes upon recognition of cognate pMHCI in a focused manner, requiring polarisation of lytic machinery to destroy the target cells (Kägi et al. 1994; Lobe et al. 1986; Faroudi et al. 2003; Pasternack & Eisen 1985).

CTL can also release large amounts IFN-γ cytokine which further increases MHCI upregulation (Seliger et al. 2008) and mediates inhibition of tumour-induced angiogenesis (Prévost-Blondel et al. 2000; Qin et al. 2003). Cell mediated cytotoxicity is a rapid and low threshold process while IFN-γ production is prolonged and requires strong antigen stimulation (Wiedemann et al. 2006). As few as two cognate pMHCI interactions at the cellular interface have been shown to be sufficient to activate cytotoxicity (Purbhoo et al. 2004). The lethal hit is delivered rapidly which allows each CTL to kill large numbers of targets either serially (Isaaz et al. 1995) or by simultaneously engaging multiple targets (Wiedemann et al. 2006). CD8+ CTL are typically more potent lytic effectors than CD4+ CTL even though both contain equal amounts of lytic granules (Beal et al. 2008). CD8+ CTL were found to have a greater ability to accumulate lytic granules into the centre of the lytic synapse whereas in CD4+ CTL the lytic granules are located peripheral to the synapse (Beal et al. 2009). These differences were related to faster calcium mobilisation in CD8+ CTL after cognate antigen stimulation thus allowing granules to be delivered to the microtubule organising centre and subsequent polarisation to occur (Sykulev 2010).

1.5.2 The role of CD4+ T cells in tumour immunity

A critical role for CD4+ T cells in inducing tumour immunity has been demonstrated in mice depleted of or deficient in CD4+ T cells (Lin et al. 1996; Hock et al. 1991; Fearon et al. 1990). The mechanisms by which CD4+ T cells promote tumour immunity are numerous (Pardoll & Topalian 1998). Perhaps the most important role is the ability of CD4+ cells to augment CD8+ T cell responses (Castellino & Germain 2006). CD4+ T cells are crucial during the activation and effector phases of tumour specific CD8+ T cells within the tumour microenvironment (Schietinger et al. 2010). The induction of a long-lived tumour specific IFN-γ producing CD8+ response has been demonstrated to require the priming of CD4+ tumour specific T cells through vaccination with MHCII peptides (Knutson et al. 2001). In other studies, co-transfer of melanoma specific CD4+ and CD8+ T cells (Rosenberg & Dudley 2004; Dudley et al. 2005) were found to be more effective than previous protocols using only CD8+ T cells (Dudley et al. 2001). A significant component of CD4+ T cell help in the tumour setting occurs via the paracrine production of IL-2 (Fearon et al. 1990), and through CD40-CD40L engagement via APC or directly with T cells (Shafer-Weaver et al. 2009). Local effects of IL-2 lead to upregulation of cytolytic function in tumour specific CD8+ T cells (Bos & Sherman 2010). The use of immunotherapy by systemic administration of IL-2 and agonistic CD40 antibodies to replicate the CD4+ T cell help effects had some short-term benefits but led to detrimental secondary responses (Berner et al. 2007). The anti-tumour effects of IL-2 and CD40 antibodies were found to be dependent on IFN-γ, however systemic administration of IFN-γ led to apoptosis of CD4+ T cells. Another approach substituted CD4+ T cell help by inducing

activation of dendritic cells in the tumour environment through administration of the TLR3 agonist polyI:C (Hervas-Stubbs et al. 2007). Although this resulted in an increase in CD8+ effectors in the periphery, without chemokine-secreting tumour specific CD4+ T cells, CD8+ effectors were not recruited into the tumour environment (Bos & Sherman 2010). This may suggest that CD4+ T cells may be better able to persist within the tumour milieu better than CD8+ T cells.

Even though most tumour cells do not normally express MHCII, CD4+ T cells are able to upregulate MHCII on cancer cells for direct recognition (Xie et al. 2010; Muranski et al. 2008). There have been historical observations that CD4+ T cells are able to target tumour cells directly (Greenberg et al. 1981). The mechanisms by which TCR transgenic CD4+ T cells can eradicate established tumour cells have been shown in detail by two recent studies (Xie et al. 2010; Quezada et al. 2010). These tumour-specific cytotoxic CD4+ T cells had features characteristic of effector CD8+ T cells expressing perforin, granzyme, CD107 and Th1 cytokines. Endogenous CD8+ T, B, NK, and NKT cells together with endogenous IFN-γ producing cells were not required for the anti-tumour effects, however IFN-γ production by CD4+ T cells was crucial (Quezada et al. 2010).

The effects of IFN-γ may extend beyond the tumour into the surrounding stromal cells (Qin & Blankenstein 2000; Muranski et al. 2008) explaining the observation that CD4+ T cells can eradicate MHCII deficient tumours in the absence of CD8+ T cells (Greenberg et al. 1985; Frey 1995; Monach et al. 1995). Cytokines secreted by tumour specific CD4+ T cells have been shown to activate macrophages and eosinophils (Hung et al. 1998; Corthay et al. 2005). A recent paper showed that CD4+ T cells were more efficient at eradicating tumour than CD8+ T cells because of the additional ability to engage stromal cells through MHCII (Perez-Diez et al. 2007). This finding was supported by a different study demonstrating that aggressive tumour can be eradicated by just targeting tumour stroma with Ag-specific CD4+ T cells (Schietinger et al. 2010). The bystander killing of tumour by targeting stromal cells required cooperation between CD4+ and CD8+ T cells (Schietinger et al. 2010).

1.6 Cancer immunotherapy using adoptive T cell transfer

1.6.1 Non-modified T cells

The earliest example of using T cell transfer to eradicate tumour is now also known as the graft-versus-leukaemia (GVL) effect (Odom et al. 1978). The establishment of GVL requires allogeneic haemopoietic stem cell transplantation (HSCT) with or without subsequent donor lymphocyte infusion/s which is now used routinely to treat human leukaemia and lymphoma (Collins et al. 1997). The basic mechanism of the

GVL effect is the recognition of minor histocompatibility antigen (HLA) mismatch in leukaemic cells by donor T cells. As the minor-HLA mismatch also occur frequently in normal tissue it is difficult to separate the beneficial GVL effect from the adverse graft-versus-host-disease (GVHD) effects. Researchers have therefore attempted to refine adoptive T cell transfer by identifying antigen specific T cell clones, which only target disease associated anitgens (eg TSA or TAA). The early successful examples of adoptive transfer using donor CD8+ CTL lines post Allo-HSCT was for CMV disease and EBV associated post-transplant lympho-proliferative disease (PTLD) (Riddell et al. 1992; Rooney et al. 1995).

Utilizing CD8+ CTL clones against tumour antigens in the autologous setting is a much more challenging proposition. Firstly, tumour-specific CTL clones have to be sourced from TILs. Although TILs have been found in patients with breast cancer (Ruffell et al. 2012), colon cancer (Ogino et al. 2011) and melanoma, only melanoma lesions have reproducibly yielded therapeutic TILs. The reasons are obscure but may be related to the high degree of mutations found in melanoma (Walia et al. 2012). Recent analysis of melanoma TIL cultures identified that the majority of specificities were directed against cancer testis or differentiation antigens (Andersen et al. 2012). Using a high throughput method to analyse melanoma TILs against 145 epitopes, Kvistborg et al found that TIL cell products from individual patients contained unique patterns of reactivity (Kvistborg et al. 2012).

Adoptive immunotherapy using autologous T cells is technically challenging and involves the isolation of TILs from melanoma lesions, in-vitro expansion and functional characterisation of the T cells. Adoptive transfer of melanoma-specific CTL clones typically follows a lymphodepleting conditioning regimen and short term IL-2 cytokine administration (Restifo et al. 2012). Such an approach has been uniquely successful in the management of melanoma, with the adoptive transfer of CTL clones in three separate trials involving 93 patients resulted in 20 patients (22%) having complete remission of 5 years or more (Rosenberg et al. 2011).

1.6.2 Genetically modified T cells

T cells can be redirected to target tumour through gene transfer of tumour-specific TCR or chimeric antibody receptors (CAR). This form of gene therapy has the potential to redirect any T cell against any cancer epitope. To date the range of tumour types that can be targeted include melanoma, leukaemia, lymphoma, sarcoma and neuroblastoma (Morgan et al. 2006; Robbins et al. 2011; Savoldo et al. 2011).

The proof of principle that TCR gene transfer could be used to redirect T cells against TAAs was first shown more than a decade ago (Stanislawski et al. 2001). The TCRα and TCRβ genes is usually first isolated from high avidity T-cell clone and cloned into

lenti- or retro-viral vector which is used to transduce polyclonal T cells (Fig 1-6). The most commonly used retroviral vectors are the moloney sarcoma cell virus (MSCV) derived vectors as these induce stable DNA integration with high efficiency (Kieback & Uckert 2010). However, transduction with retroviral vectors requires T cell activation which may lead to reduce in-vivo functional activity. Alternatives to MSCV vectors include lentiviral, transposon based and zinc finger nuclease vectors which either improve the safety profile by reducing the risk of insertional mutagenesis or allow gene insertion into quiescent T cells without prior activation.

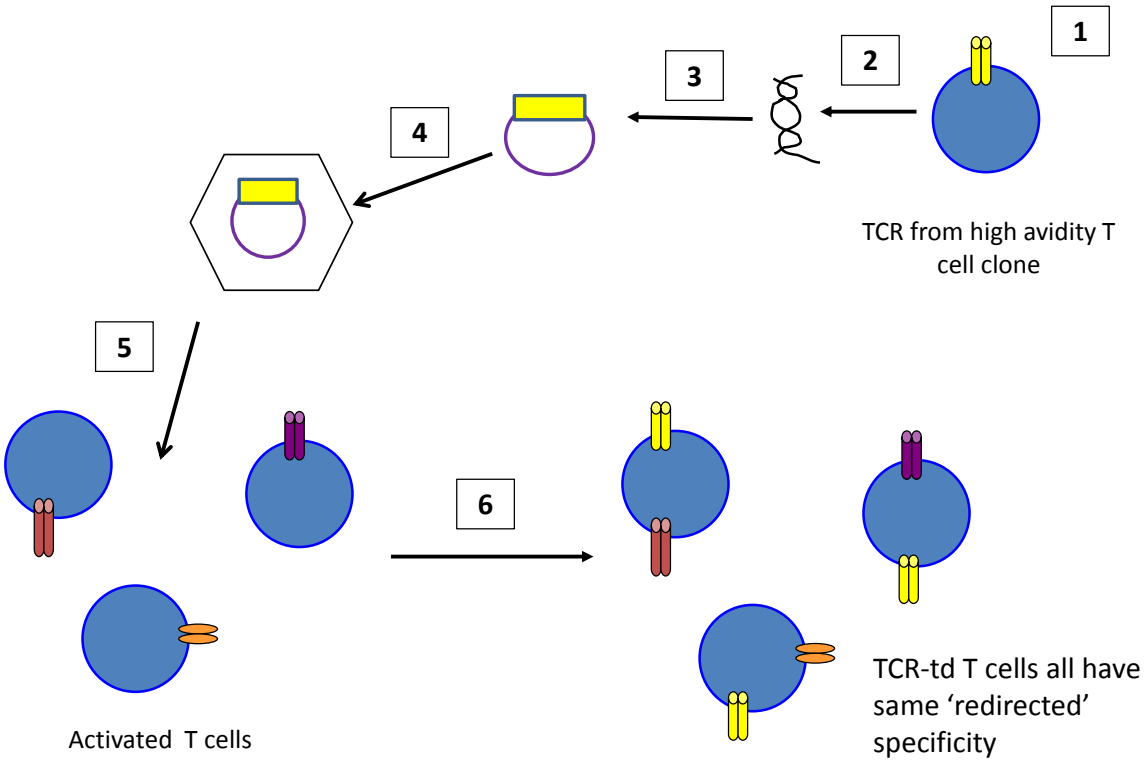


Figure 1-6 Schematic diagram showing the different stages of TCR gene transfer. 1.) Isolation of high avidity T cell clone (lymphocyte represented by blue round cell with TCR represented by yellow dimer). 2.) Determination of TCR genes from T cell clone (black double helix structure). 3.) Cloning of TCR genes into retroviral vector (purple line). 4.) Transfection of the retroviral vector into phoenix packaging cells which produce retroviral particles (represented by hexagonal shape surrounding vector). 5.) Transduction of activated T cells (polyclonal TCR represented by other non-yellow dimers) using supernatant containing retroviral particles. 6.) Confirm the expression of transduced T cells by FACS (transduced cells express introduced TCR represented by yellow dimer).

The TCR genes are isolated from T cell clones with high avidity for the relevant Ag. Previously, when the TCRα and TCRβ genes were cloned into two separate transfer vectors, transduction efficiency was suboptimal. Relatively recent advances in vector technology have developed the use of internal ribosome entry site (IRES) or picornavirus-derived 2A linker peptides (Furler et al. 2001) to enhance equimolar expression of the two TCR chains cloned into bicistronic viral vectors. Some transgenic TCRs are expressed poorly on human cells but can be overcome by optimising the mRNA sequence for translation in the human host using a process known as codon optimisation.

It is known that the introduced α and β chains may mispair with endogenous β and α chains, respectively, in transduced T cells, resulting in potentially deleterious/auto-reactive new specificities (Bendle et al. 2010). Strategies to reduce this involve

structural modifications such as the introduction of new cysteine residues at position 48 of the α-chain and position 57 of the β-chain constant regions. This modification permits the formation of an additional disulphide bond at a unique site between the introduced TCR chains (Boulter et al. 2003; Cohen et al. 2007). Another structural modification whereby replacing the human TCR constant region sequences with murine constant region sequences to form a human-murine hybrid TCR not only reduces mispairing but also increases introduced TCR expression at the cell surface (Cohen et al. 2006). The most recent approach utilised to reduce mispairing is the knock down of endogenous TCR expression using siRNA constructs at the same time as introducing tumour specific TCR (Kuball et al. 2007; Nagai et al. 2011).

The specificity and functional avidity of TCR transduced (TCR-td) T cells to the redirected target is determined by properties intrinsic to the introduced TCR such as affinity for pMHC and 'strength' as determined by the ability to outcompete endogenous TCR for expression (Stauss et al. 2007). TCR isolated from T cells taken from nontolerogenic environments were found to be more effective against TAA which as elaborated in Section 1.5 are self-Ag. This is not only because high avidity T cells are not deleted and hence are available for isolation and also because tolerance may be regulated proximally at the level of the TCR and TCR transduced T cells do not become anergic in the presence of TAA (Teague et al. 2008). Nontolerized T cells are screened from MHC mismatch lymphocytes but are still required to be able to recognize TAA presented by host MHC, a concept known as allo-MHC-restriction. Using this approach high avidity human T cell clones against cyclin-D1, WT1 and MDM2 have been isolated. TCR genes from these high avidity T cells are then cloned into vectors for gene-therapy as shown in Fig 1.6.

The functional avidity of TCR-td T cells can be improved by introducing molecular modifications to alter the TCR structure, which may improve antigen sensitivity or increase the level of TCR expression (Fig 1-7). Several approaches have been described; codon optimisation of the TCR sequence for optimal translation of RNA (Hart et al. 2008; Scholten et al. 2006), increasing the amount of available CD3 molecules within the transduced T cell through the co-transfer of CD3 γ, δ, ε and ζ chains (Ahmadi et al. 2011), removal of TCR N-glycosylation (Kuball et al. 2009a) and modification of the framework CDR region (Robbins et al. 2008).

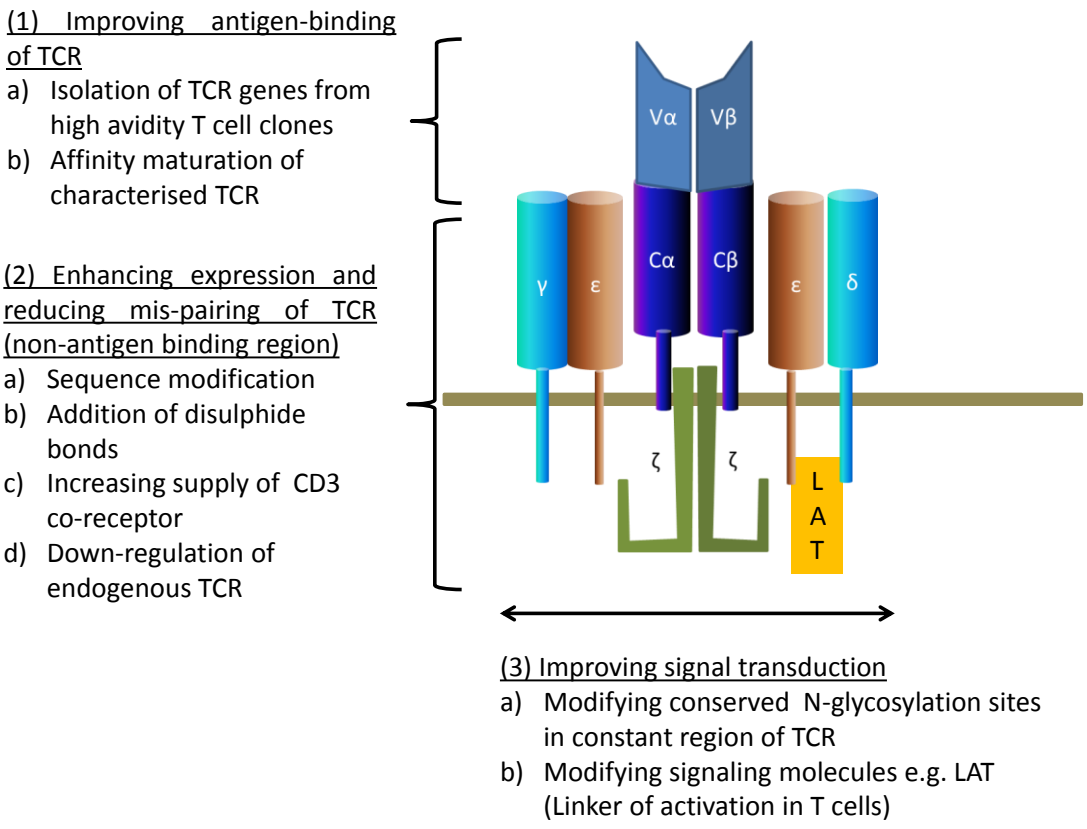


Figure 1-7 Molecular engineering of TCR signalling complex can improve T cell avidity.
Using TCR/CD3 complex annotated in Fig 1.1, modifications are grouped into three topographical regions using TCR structure anotated in Fig 1-1; (1) TCR antigen-binding site (VαVβ) (2) TCR framework regions and (3) Components relevant to signal transduction.

Tumour specific TCR-td CD8+ T cells have the ability to kill a broad range of tumour cell lines and tumour cells from patients (Stanislawski et al. 2001; Sadovnikova & Stauss 1996; Gao et al. 2000; Amir et al. 2011). Proof of principle studies have shown that TCR-td T cells can eradicate both mouse and human tumours in mouse models (Schumacher 2001; Xue et al. 2005; Xue et al. 2010). The first clinical use of TCR-td T cells was published in 2006 by Morgan et al (Morgan et al. 2006); 15 patients with metastatic melanoma were infused with autologous T cells transduced with a TCR specific for the melanoma associated peptide MART-1, a melanoma differentiation antigen. Two out of 15 patients (13%) showed full clinical regression of metastatic melanoma, with transferred cells persisting in the circulation for more than a year. A second trial targeting synovial cell carcinoma and melanoma using a TCR recognising NY-ESO-1 a cancer testes antigen had tumour shrinkage in five out of eleven patients (Robbins et al. 2011).

1.6.2.1 Chimeric Antigen Receptor (CAR) gene transfer

Effective targeting of tumour has also been performed with CAR-modified T cells. The chimeric antigen receptor (CAR) is an artificial molecule consisting of a single chain immunoglobulin based antigen-binding site fused to a CD3ζ domain (Fig 1-8). The antibody-based receptor is able to bind to surface molecules such as CD19 with high affinity but cannot recognise peptides derived from intracellular proteins presented by MHC molecules. Early clinical trials using CAR-transduced T cells showed a poor level of persistence of these cells after transfer (Park et al. 2007; Kershaw et al. 2006). However advances in molecular engineering of CAR with the addition of CD28 or CD27 co-stimulatory domains have improved the viability of transferred cells (Savoldo et al. 2011; Song et al. 2011). Several clinical trials have been published with the use of CAR-transduced T cells directed against CD19 and/or CD20 positive B-cell malignancies (Till et al. 2008; Savoldo et al. 2011; Heslop et al. 2010) and neuroblastoma (Pule et al. 2008). In one of these studies, of the eight patients given CAR-transduced T cells, six patients had remission of disease (Kochenderfer et al. 2011).

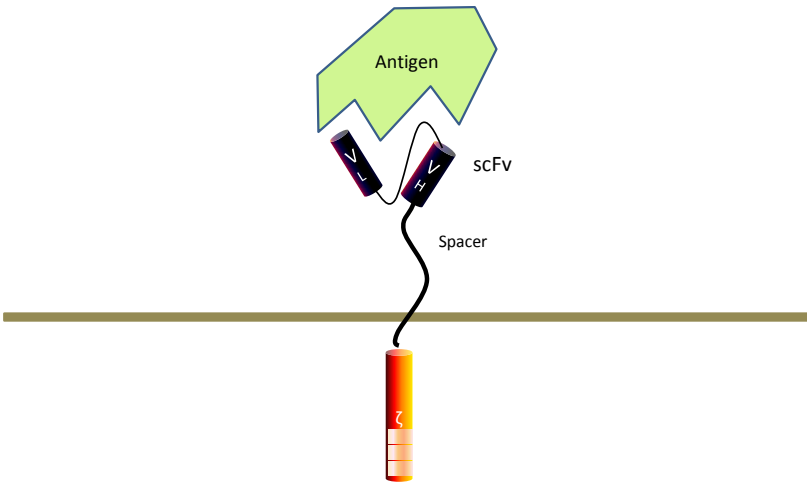


Figure 1-8 Schematic diagram showing the structure of chimeric antigen receptor (CAR).
Unlike TCR (Fig 1-4), CAR bind to conformational epitopes on non-MHC antigen and is linked to a cytoplasmic ζ-chain molecule (orange) containing ITAMS (white stripes) within ζ-chain.

Some of the TAAs targeted by CAR are expressed in normal tissues and the cross-reactivity has resulted in toxicity; this is also known as 'on-target' toxicity. For example when the CAR with specificity for the TAA carbonic anhydrase IX (CAIX) was used to treat renal cell carcinoma, the first three patients developed liver enzyme disturbances as a result of cross reactivity with bile duct epithelial cells which also express CAIX (Lamers et al. 2006). In a separate study using anti-ERBB2-CAR transduced T cells to treat colonic carcinoma, one patient developed fatal lung

inflammation complications thought to be related to low levels of ERBB2 expression on epithelial cells (Morgan et al. 2010).

1.7 Project background

There is growing evidence that the efficacy of adoptive T cell immunotherapy is enhanced when tumour-specific CD8+ and CD4+ T cells are infused together (Kuball et al. 2005; Morris et al. 2005; Restifo et al. 2012). The exact mechanisms may be related to the ability of CD4+ T cells to augment the functional avidity, expansion and persistence of cytotoxic CD8+ T cells. CD4+ T cells also known as T-helper (Th) cells provide 'help' for the generation of CD8+ T cell effector and central memory responses (Bevan 2004). Furthermore, there is growing evidence that CD4+ T cells may kill cancer cells through direct tumour cytotoxicity, indirectly by targeting tumour-related stromal cells (Section 1.5.2) and rescue self-antigen tolerant tumour specific CD8+ cells (Shafer-Weaver et al. 2009).

It has been difficult to date to isolate high avidity tumour-specific CD4+ T cells (Wang 2001). The majority of tumour cells do not express MHCII or co-stimulatory molecules (Hermans et al. 1998; Staveley-O'Carroll et al. 1998; Shrikant et al. 1999). We have shown that MHCI restricted TCR can be transferred into CD4+ cells resulting in MHCI-restricted helper T cells (Morris et al. 2005). There are examples in the literature of CD8-independent MHCI restricted TCR which when transduced into CD4+ T cells are able to elicit Ag-specific responses such as IL-2, IFN-γ secretion and cytotoxicity (Chhabra et al. 2008; Ray et al. 2010; Engels et al. 2012). These include a TCR targeting the WT1-derived peptide (RMFPNAPYL) and an affinity-matured TCR targeting NY-ESO-1 peptide both presented by HLA-A2 (Tsuji et al. 2005; Robbins et al. 2008). However, some MHCI restricted TCR-td CD4+ T cells had a deficit in cytokine production (Morris et al. 2005) or were unable to recognise tumour cells endogenously expressing cognate Ag (Moore et al. 2009). The impairment of these transduced CD4+ T cells may have been due to deficits in TCR signalling. It is possible that these high affinity TCRs are unable to detect low antigen density on tumour cells because serial triggering is suboptimal (Valitutti et al. 1995; Thomas et al. 2011). As the CD8 co-receptor has been shown experimentally to enhance TCR serial triggering (Viola et al. 1997), dysfunction of MHCI restricted TCR-transduced CD4+ T cells may be related to absence of CD8 co-receptor.

Perhaps it is surprising that the strategy of enhancing MHCI restricted TCR in CD4+ T cells by utilising the CD8 co-receptor has only been reported by a three groups (Willemsen et al. 2005; Morris et al. 2005; Kessels et al. 2006). The earlier papers from 2005 by Williamsen and our group co-introduced the CD8α gene with TCR into CD4+ T cells. The in vitro data from Willemsen showed that the MART-1 specific TCR transduced CD4+ T cells were able to produce antigen-specific responses in the form

of IFN-γ, TNF-α and IL-2 secretion when the CD8α gene was introduced. Our group showed that F5-TCR-td murine CD4+ T cells were able to produce IL-2 but not IFN-γ in response to cognate Ag and this pattern was changed when CD8α was co-transduced with co-transduced CD4+ T cells producing IFN-γ but smaller amounts of IL-2. To date, only Kessels et al have utilized both the CD8α and CD8β gene to generate CD8αβ+ TCR-transduced CD4+ T cells and showed convincing data of augmentation of antigen-specific CD8+ T cell function after invivo influenza-A challenge in mice. The results of these three studies are summarised in Table 1-1.

	Williamsen et al, 2005	Morris et al, 2005	Kessels et al, 2008
MHCI restricted TCR	Chimeric MAGE-A1 TCR	F5-TCR	OT1-TCR
CD8 co-receptor	Human CD8α	Murine CD8α	Murine CD8αβ
In vitro data of TCR-td CD4+ T-cells	No staining with HLA-A1/MAGE tetramer. Low levels of cytotoxicity against MAGE+/A1+ melarnoma.	Positive staining with NP tetramer. Produce IL-2 but not IFN-γ against EL4-NP tumour cells. Undergo proliferation in presence of EL4NP tumour cells	Produce low levels of IL-2, IFN-γ and CD40L against splenocytes loaded with OT1 peptide.
In vitro data of TCR and CD8 co-transduced CD4+ T cells	Positive staining with HLA-A1/MAGE tetramer. Moderate levels of cytotoxicity against MAGE+/A1+ melarnoma. Produce IFN-γ, TNF-α, and IL-2 with MAGE+/A1+ melanoma	Positive staining with NP tetramer. Produce IFN-γ but lower levels of IL-2 against EL4NP tumour cells. No proliferation in presence of EL4NP tumour cells	Produce higher levels of IL-2, IFN-γ and CD40L against splenocytes loaded with Ova257 peptide.
In vivo function of TCR-td CD4+ T cells	Not assessed	Good tumour protection against EL4NP tumour cells only in the presence of F5-TCR transduced CD8+ T-cells. 80% of mice survive tumour challenge.	Did not induce expansion of endogenous CD8+ against influenza-A detected by NP366 tetramer, after MHCII deficient mice were challenged with Influenza A containing ova.
In vivo function of TCR and CD8 co-transduced CD4+ T cells	Not assessed	Poor tumour protection against EL4-NP tumour cells even in the presence of F5-TCR transduced CD8+ T-cells. Only 20% of mice survive tumour challenge.	Did not induce expansion of endogenous CD8+ against influenza-A detected by NP366 tetramer, after MHCII deficient mice were challenged with Influenza A containing ova.

Table 1-1 Summary of results taken from three studies co-transducing CD8 co-receptor with MHCI restricted TCR into CD4+ T cells.

The same paper by Kessels et al also explored mutating CD8 co-receptors to enhance T cell avidity. They modified the intra-cytosolic signalling domain of the CD8α chain with the sequences from the CD4 co-receptor signalling domain which allowed increased Lck to bind with the aim of mediating greater signal transduction (Wiest et al. 1993; Shaw et al. 1989; Erman et al. 2006). However, the in vitro studies with CD4+ T cells co-transduced with the mutant CD8 co-receptor and OT1 TCR did not improve function compared to cells expressing the wild-type CD8 co-receptor.

Another hypothetical way to augment the CD8 co-receptor is by increasing its affinity to MHCI. Anti-CD8 antibodies such as from the murine anti-CD8α 53.6.7 and human anti-CD8α OKT8 have been known to increase multimer staining of murine and human T cells (Daniels & Jameson 2000; Campanelli et al. 2002). The anti-CD8α antibody 53.6.7 bind to the T81 residue of the CD8α chain and does not direct contact MHCI (Devine et al. 2004). Devine et al therefore postulate that the improvement of MHCI affinity may be due to a more stabilised CD8 conformation. However there is no published data using the anti-CD8α antibody 53.6.7 to improve T cell function. The human anti-CD8α OKT8 however increased the antigen specific production of MIP-1β by T cell line 3G10 (Wooldridge et al. 2003). Wooldridge et al also studied the effects of increasing CD8 affinity by introducing the Q115E mutation to the α2 domain of HLA-A2 and found that peptide loaded APC harbouring HLA-A2 Q115A molecules could significant increase the cytokine production and proliferation of naïve T cells (Laugel et al. 2007).

1.8 Aim of the project

This project aims to test mutations in the CD8 co-receptor for augmentation of effector function of genetically modified T cells expressing a tumour-specific TCR. CD8 co-receptor mutations that are shown to enhance T cell avidity in vitro will be tested in an in-vivo mouse model for the ability to afford improved tumour protection. The ability to augment the avidity of genetically modified T cells for cancer immunotherapy is an attractive proposition as most anti-tumour T cells are of low avidity (Uttenthal et al. 2012). Moreover an augmenting CD8 co-receptor could potentially improve the function many MHCI-restricted CD8 dependent TCR. Whereas modifications of the murine CD8α chain did not influence, in either a positive or negative manner, Kessels et al (Kessels et al. 2006) found that the CD8β was critical for the function of MHCI restricted OT1-TCR in CD4+ T cells. Around the same time, Devine et al (Devine et al. 2006) analysed 23 murine CD8β chain mutants in the CDR1, CDR2 and CDR3 loops and found both four CD8β mutants (S53L, S54V, L58R and L58R/I25A) that enhanced binding to MHCI molecule H2-Kb compared to wild-type CD8β. The CD8α chain and CD8β chain containing mutations were transfected

into COS-7 fibroblast. The transfected COS-7 cells were stained either with CD8α antibody, CD8β antibody or H2-Kb tetramer. The binding index was calculated based on the relative tetramer MFI to CD8β MFI of transfected cells. Two of the CD8β mutants S53L and I25A/L58R were transduced into T cell line expressing 2C-TCR and CD8α and found to enhance IL-2 production to peptide loaded RMAS target cells.

The candidate mutations selected for investigation were within the CD8β chain and consisted of two categories. The first group of mutations were introduced into the MHC-binding region (S53L, S54V, L58R and L58R/I25A) previously identified by Devine et al as described above. The second group of mutations involved O-glycosylation sites (T120A, T121A, T124A), following previous studies, which had demonstrated that the removal of these threonine sites prevented O-glycosylation sialylation. These mutations achieved a default desialylated state, which has been shown to enhance T cell avidity (Section 1.4.2).

Chapter 2

Chapter 2. Materials and Methods

2.1 Molecular techniques

2.1.1 Retroviral vector backbones

The pMX vector encodes the Moloney murine leukaemia virus (MLV) long terminal repeat (LTR) sequences, subgenomic envelope (env) and gag sequences which facilitates encapsidation and expression of inserted sequences. Protein coding sequences were inserted between the NotI and EcoRI sites. The pMP71 vector is a second generation retroviral vector with the MPSV LTR containing 5' untranslated sequences derived from the murine embryonic stem cell virus (MESV). The pMP71 vector has been shown to enhance transgene expression by more than ten times compared to the MLV based vectors (2003 HGT Uckert) and was a kind gift from Dr Wolfgang Uckert (Institute of Biology, Humbult-University Berlin).

Details of the cloning strategy used to generate specific retroviral vectors used in this project are given in Chapter 3.

2.1.2 Bacterial culture

2.1.2.1 Preparation of LB broth and agar

To prepare 10 litres of LB broth, 250 g of LB broth (Fisher Scientific, Loughborough, UK) was dissolved in 10 litres of distilled H_2O. Broth was aliquoted into 500 ml bottles and autoclaved prior to use. Ampicillin (0.1mg/ml) was added to the LB broth and mixed well before use under sterilizing conditions.

To prepare 8 litres of LB agar, 200 g of LB broth and 60 g of LB agar (Fisher Scientific, Loughborough, UK) were dissolved in 8 litres of distilled H_2O. Broth was aliquoted into 400 ml bottles and autoclaved prior to use.

To prepare 10 plates of LB agar containing ampicillin, 400 ml of LB agar in a bottle was heated in a microwave until agar was fully melted and left to cool. 4 mg of Ampicillin was added to the liquid agar and mixed well before plating out under sterilizing conditions.

2.1.2.2 Transformation of competent bacterial cells

For transformation, DH5α competent bacterial cells (Invitrogen) were thawed on ice and divided into 50 µl per tube. These were then incubated on ice for ten minutes. The 10 µl ligation reaction or low concentration plasmid (10 ng/µl) was then mixed with the DH5α. These were placed onto ice for a further 30 mins, before heat shock at 42°C for 30 seconds and then placed back onto ice for a further two minutes. Five volumes of SOC medium (Invitrogen) was then mixed with the bacteria and shaken at 220 rpm at 37°C for one hour. After this time, the bacterial cells were spread onto

LB agar plates containing 0.1 mg/ml ampicillin (Sigma-Aldrich). The plates were incubated at 37°C overnight until colonies were visible. The plates were stored at 4°C for up to one week before colonies were picked.

2.1.3 Large scale preparation of plasmids

A single colony from LB agar plates was picked and inoculated into 2-5 mls of LB broth containing 0.1mg/ml ampicillin then shaken at 220 rpm at 37°C for 6-10 hours. Glycerol stocks for each sample were prepared at this stage by mixing 300µl of the bacterial culture with 300µl sterile glycerol (Sigma-Aldrich) and storing at -80°C. After 6-10 hours of shaking when the inoculated LB broth became cloudy, 0.1ml of the starter culture was then inoculated into 100ml (1:1000 dilution) of LB broth containing 0.1 mg/ml ampicillin and then shaken at 220rpm at 37°C overnight for 12-16 hours. Plasmid DNA was extracted using a Midiprep or Maxiprep Kit (QIAGEN 12143/12163), following the protocol supplied with the kit. Typically, 50-500 µg of plasmid DNA was recovered. The DNA pellet was re-dissolved in the required volume of low concentration Tris-EDTA (LCTE) buffer to give a final DNA concentration of 1 µg/µl.

2.1.4 PCR amplification

This technique was used primarily to introduce unique restriction sites on the 5' and 3' ends of DNA fragments containing the gene of interest to allow insertion into the retroviral vector backbones described in Section 2.1.1. Forward and reverse primers containing the desired restriction and annealing sequences were designed using Netprimer software (premierbiosoft). This programme analyses the primer secondary structures and determines the predicted melting temperatures. The PCR solution contained the following: Pfu-Mix containing 2mM dNTP and 10x Buffer (66 µl), Pfu (2 µl), forward and reverse primers (6 µl each), DNA (gene construct or negative control) 10 ng/µl (6 µl), and H_2O (8 µl). 36 cycles of PCR were performed. The PCR product was checked for the presence of the amplified gene construct by analysis of PCR products on a1% agarose gel, alongside a 200bp to 10kb HyperLadder I (Bioline).

2.1.5 PCR mutagenesis

Oligonucleotide primers were designed to contain nucleotide changes at the desired position within the gene construct using Stratagene primer design software and shown in Chapter 3 Table 3-2. The primers were synthesized and purified by Invitrogen. PCR mutagenesis was performed utilizing these primers and the retroviral vector containing gene construct using the QuikChange XL II site directed mutagenesis kit (Strategene 200521). XL-10 gold ultracompetent bacteria were

transformed using the PCR product as described using kit protocol. DNA was extracted by using QIAprep Miniprep Kit (QIAGEN 27106) as described above.

2.1.6 Restriction digestion and gel extraction

Restriction digests were performed to separate the gene construct from vector. 1µg of miniprep DNA was digested for 1-2 hours at 37°C with the appropriate restriction endonuclease and buffer. The restriction enzymes (NotI, XhoI, RsrII, BstZ17I and EcoRI) were obtained from New England BioLabs. Digested DNA samples were then separated on a 1% agarose gel containing 0.2 µg/ml Ethylene bromide (Sigma-Aldrich), alongside a 200bp to 10kb HyperLadder I (Bioline). The desired DNA fragment was isolated by gel extraction and PCR purification (QIAGEN 28106).

2.1.7 Ligation

The ligation of genes (inserts) into the linearized pMP71 vector backbone was performed using a 10µl reaction containing 1µl 10x T4 DNA ligase buffer (New England BioLabs), 0.5µl (200U) T4 DNA ligase (New England BioLabs), 30-50 ng of insert and 10-30 ng of linearized pMP71 at a molar ratio of 3-6:1. The reactions were incubated at 14°C overnight. Samples were then separated on a 1% agarose gel containing 0.2 µg/ml Ethylene bromide (Sigma-Aldrich E1510), alongside a 200 bp to 10kb HyperLadder I (Bioline).

2.1.8 Sequencing of DNA

Plasmid DNA was sequenced at Beckman Coulter Genomics, Takeley, Essex. Sequencing of gene constructs in the pMP71 vector was performed using appropriate primers. DNA sequences were analysed using an open source programme, A Plasmid Editor (APE) (http://www.biology.utah.edu/jorgensen/wayned/ape/).

2.2 Cells, Cell lines, and culturing conditions

All cell culture work was performed under strict sterile conditions in tissue culture hoods to minimize and prevent infection from microbes.

2.2.1 Media

Cell culture growth media were based on IMDM (Lonza BE12-722F) or RPMI 1640 (Lonza BE12-167F) with addition of 100 U/ml penicillin and 100µg/ml streptomycin (Gibco 15070), 2mM L-glutamine (Gibco 25030) and 50 µM 2-mercaptoethanol (Sigma M6250) and 10% foetal calf serum (FCS). For cell lines, heat inactivated South American Origin FCS was used (Biosera S1810). Validated batches of heat-inactivated FCS were added to RPMI medium used for the culture of primary murine splenocytes/T cells (Sigma F7524) and human PMBC/T cells (Gibco 10270).

2.2.2 Antigen presenting cells and tumour cell lines

RMAS cells (H2b) are derived from a Rauscher virus-induced C57BL/6 T cell lymphoma and are TAP-deficient due to a point mutation in the TAP2 gene. RMAS were used as antigen presenting target cells for murine T cells by overnight temperature induction at 25°C and loaded with exogenous peptides for 2 hours. RMAS cells were split every 2-3 days 1:8 with fresh RPMI cell line medium and kept humidified at 37°C and 5% CO_2.

HLA-A2+ T2 cells are also a TAP-deficient cell line and used as antigen presenting target cells for human CD4+ T cells by loading with exogenous peptides for 2 hours at 37°C. T2 cells were split every 2-3 days 1:8 with fresh RPMI cell line medium and kept humidified at 37°C and 5% CO_2.

Fresh murine splenocytes harvested from C57BL/6mice (H2b) were used as antigen presenting target cells for murine CD4+ T cells (Chapter 4) and were loaded with exogenous peptide for 2-6 hours.

EL4 cells are a murine lymphoma cell line which expresses H2-Db MHCI and do not express any MHCII molecules. EL4 cells that stably express the Influenza-A virus derived nucleoprotein (EL4NP) were a kind gift from Dr B Stockinger (National Institute of Medical Research, Mill Hill, London) and were used as target cells for both murine CD8+ and CD4+ T cells as discussed in detail in Chapters 4 and 5. In addition, EL4NP cells were transfected with a luciferase plasmid (EL4NPluc), which was a kind gift of Dr M Pule (University College London, UK). These cells were used for in vivo monitoring of tumour growth by bioluminescence as described in Chapter 6. EL4, EL4NP and EL4NPluc cells are split every 2-3 days 1:8 with fresh RPMI medium and kept humidified at 37°C and 5% CO_2.

2.2.3 Retroviral packaging cell lines

Phoenix-Ecotrophic (PhEco) cells (Orbigen, Santa Cruz labs) are retroviral packaging cells capable of producing *trans* proteins (env, gag and pol) that are required for the packaging, processing, reverse transcription, and integration of recombinant genomes. Viral envelope particles produced using PhEco cells have tropism for murine cells due to expression of mouse cationic aa transporter (mCAT1). For optimal transfection, fresh aliquots of 3-5 $x10^6$ PhEco cells were thawed and seeded onto the flat side of T75 tissue culture flasks (TPP 90076) with fresh IMDM medium. Each batch of PhEco cells were maintained in continuous culture for a maximum of four weeks. For maintenance, sub-confluent PhEco cells were split by 1:6-1:8 with EDTA/Trypsin (Gibco 25300) into a new T75 flask every 2-3 days and kept humidified at 37°C and 5% CO_2.

Phoenix-Amphotrophic (PhAmpho) cells are retroviral packaging cells similar to PhEco but the viral envelope protein recognizes the amphotrophic receptor RAM1 a phosphate transported found on human cells. For optimal transfection, fresh aliquots of 3-5 x10^6 PhAmpho cells were thawed and seeded onto the flat side of T75 flasks with fresh IMDM medium and used for transfection for up to four weeks after thawing. For maintenance, sub-confluent PhAmpho cells were split 1:6 with EDTA/Trypsin (Gibco) and seeded into a new tissue culture-treated T75 flask every 2-3 days and kept humidified at 37°C and 5% CO_2.

2.2.4 T cell lines

The 58α-β- (BW) T cell line are CD8+ TCR- cells, a variant of the D0–11.10 T cell hybridoma and were used for TCR transfer experiments. CD8β negative BW cells expressing only CD8α were used to validate co-transfer of TCR and CD8β molecules. For maintenance, BW cells were split 1:8 with fresh RPMI medium and kept humidified at 37°C and 5% CO_2.

2.3 Generation of TCR-transduced T cells

2.3.1 Transfection and production of recombinant retroviral particles

Sub-confluent PhEco or PhAmpho cells maintained in T75 flasks were removed with EDTA-Trypsin, washed with fresh IMDM medium and counted. 1.4-2.0 x10^6 PhEco/PhAmpho cells were resuspended in 8ml fresh IMDM growth medium and seeded into 60 cm^2 sterile tissue culture-treated petri dishes (TPP 93100) and kept humidified at 37°C and 5% CO_2 for 24 hours. The next day, the cell medium was changed and replaced with 5 ml fresh IMDM and kept humidified at 37°C and 5% CO_2 for 4-8 hours. Each dish containing PhEco/PhAmpho cells were then transfected, by adding drop-wise, a mixture containing 2.4µg vector (TCR or Cd8) DNA, 1.5µg pCL-Eco/pCL-Amp DNA, 50 µl dH_2O and 150 µl Opti-MEM solution (Sigma 31985) and kept humidified at 37°C and 5% CO_2 for 12-16 hours. The retroviral vectors pCL-Eco and pCL-Amp encode an ecotropic or amphotropic envelope, respectively, which are used to enhance retroviral transduction of murine/human cells (Naviaux et al. 1996). These vectors were kindly provided by Dr T Schumacher (Netherlands Cancer Institute). For mock-transfection controls, TCR-expressing vector was omitted and replaced with an equivalent volume of dH_20. The next day, IMDM growth medium was gently removed from PhEco/PhAmpho cells, without disturbing adherent cells, and replaced with fresh 5 ml RPMI T cell medium for a further 16 hours during which virus particles were produced. For optimal production of retroviral particles the PhEco/PhAmpho cells should be sub- confluent when supernatant is harvested.

2.3.2 Preparation of T cells before and after transduction

Wild-type C57BL/6 mice were bought from Harlan, UK or acquired from in-house breeding colonies maintained at UCL Medical School. The CD8β-deficient C57BL/6 mice which originated from a breeding colony maintained at the National Institute of Medical Research and were a kind gift from Dr R Zamoyska (University of Edinburgh). All procedures performed on these mice were carried out in accordance with UK Home Office regulations under an approved Project License.

2.3.2.1 Murine CD8+ T cell purification

To enrich for CD8+ T cells, 1-5 $x10^8$ splenocytes harvested from wild-type C57BL/6 mice or CD8β-deficient C57BL/6 mice were labelled with anti-CD8α MicroBeads (Miltenyi 130-049-401) and passed through LS or LD magnetized separation columns as directed by the manufacturer (Miltenyi 130-042-401, 130-042-901) and the cells eluted from the column were collected for transduction. In earlier experiments (Chapter 4), CD8β-deficient CD8+ T cells were depleted of Vβ11+ cells after staining with anti-Vβ11-FITC antibodies (BD Pharmingen) and anti-FITC MicroBeads (Miltenyi 130-048-701). The labeled cells were passed through magnetized LD column (130-042-901) and the flow through containing Vβ11 negative cells were collected for transduction.

After transduction, F5-TCR and CD8β co-transduced CD8β-/- CD8+ T cells were enriched by positive selection using anti-mouse CD8β-FITC (BD Pharmingen) and anti-FITC Microbeads (Miltenyi, 130-048-701) and re-stimulated with splenocytes and NP peptide. The re-stimulated cells were rested for 7 days before functional assays were performed (Chapter 4).

2.3.2.2 Murine CD4+ T cell purification

To deplete CD8+ T cells, 1-5 $x10^8$ splenocytes harvested from wild-type C57BL/6 mice or CD8β-deficient C57BL/6 mice were labelled with anti-CD8α MicroBeads (Miltenyi 130-049-401) and passed through magnetized LD magnetic columns (Miltenyi 130-042-901). The flow-through cells from the column were subsequently enriched for CD4+ T cells by labelling with anti-CD4 MicroBeads (Miltenyi 130-049-201) and passage through LS magnetic columns (Miltenyi 130-042-401). The CD4+ T cells eluted from the column were retained for transduction.

After transduction, F5-TCR and CD8 co-transduced CD4+ T cells were further enriched by positive selection using anti-mouse CD8α-APC (Abcam ab22504) and anti-APC Microbeads (Miltenyi, 130-048-701). The CD8+ enriched cells were used for functional assays the following day (Section 5.3.2).

2.3.2.3 Human CD4+ T cell purification

CMV-negative HLA-A2+ PBMCs from healthy donors were isolated by density gradient centrifugation of buffycoats obtained from the National Blood Service (London, United Kingdom). 2 $x10^7$ PBMCs from each donor were aliquoted and stored in liquid Nitrogen. For transduction, 4 $x10^7$ frozen PBMCs were thawed, washed and examined with tryphan-blue. To deplete CD8+ T cells, PBMCs were labelled with anti-CD8α MicroBeads (Miltenyi 130-045-201) and passed through LD magnetic columns (Miltenyi). Flow-through cells were collected and enriched for CD4+ cell enrichment after labelling with anti-CD4 MicroBeads (Miltenyi 130-045-101) as described above.

2.3.3 Transduction of T cells

2.3.3.1 Murine T cells

The sorted CD8+ or CD4+ T cells were activated for 1.5 days with mouse CD3/CD28 Dynabeads (Invitrogen 11453), 100 units/ml of IL-2 (Chiron, Emeryville, CA) and fresh Roswell Park Memorial Institute (RPMI) T cell medium (Day 0) and incubated at 37°C and 5% CO_2 for 48 hours in tissue culture plate (TPP 92006). The next day (Day 1), wells on a suspension culture plate (Cellstar 657185) were coated with 1.5ml Retronectin (Takara-Bio T100B) and incubated overnight at 4°C. The following day (Day 2), Retronectin was recovered and stored at -20°C (for up to eight repeats). For each condition, 1.5-3.0 ml of retroviral supernatant with viral particles containing TCR alone or a 1:1 mixture of TCR and CD8 constructs were mixed with 5-10 $x10^6$ T cells. The T cell and retroviral supernatant mixture was then added to the Retronectin coated wells and spun at 440G for 90 minutes. After centrifugation, the plate was kept humidified at 37°C and 5% CO_2 for 24 hours. The next day (Day 3), Dynabeads were removed and fresh medium/ IL-2 (100 U/mL) (Chiron, Emeryville, CA) was added to the splenocyte cultures. Cells were analysed by flow cytometry from day 5-7. Transduced T cells were used for functional assays from Day 6-8.

2.3.3.2 Human T- cells

The sorted CD4+ T cells were activated for 1.5 days with anti-CD3 OKT3 antibodies, 600 units/ml of IL-2 (Chiron, Emeryville, CA) and fresh RPMI T cell medium (Day 0) and incubated at 37°C and 5% CO_2 for 48 hours. The next day (Day 1), wells on suspension culture plate (Cellstar 657185) were coated with 1.5ml of Retronectin (Takara-Bio, Japan) and incubated at 4°C overnight. The following day (Day 2), Retronectin was recovered and frozen for future use (up to eight repeats). For each condition, 1.5-3 ml of retroviral supernatant with viral particles containing CMV-TCR/CD8 constructs was mixed with 5-10 $x10^6$ T cells. The Retronectin coated plate containing T cells and viral supernatant was kept humidified at 37°C and 5% CO_2 for 24 hours. The next day (Day 3) fresh medium and IL-2 (100 U/mL) (Chiron,

Emeryville, CA) were added to culture wells containing T cells. Cells were analyzed by flow cytometry from day 5-7. At day 7-10 after activation, transduced T cells were re-stimulated with PBMC, pp65 peptide and IL-2 (Roche). This process was repeated every 7-10 days for not more than four cycles to obtain a high purity of transduced cells (Chapter 5).

2.4 Flow cytometry

Samples were stained on ice in PBS (1% FCS) with the appropriate dilution of the relevant monoclonal antibodies (mAbs). Propidium iodide was used to stain dead cells. Samples were acquired on an LSR2 or Fortessa flow-cytometer (BD Biosciences), with compensation first performed using single stained lymphocytes and FACS Diva software. The following mAbs were used for flow cytometric staining: rat anti-mouse CD4-APC-H7 (BD 560181), rat anti-mouse CD8α-APC (BD 553035), rat anti-mouse CD8α-CyChrome (BD 553034) and rat anti-mouse CD8β-FITC (BD 553040). As MDM-TCR contain a c-Myc sequence tag in the TCRβ chain it was detected using mouse anti-c-Myc (A-14) (Santa Cruz Biotechnology sc-789) and anti-mouse IgG1 PE (BD 550083) mAbs were used to determine TCR expression. The F5-TCR utilizes Vβ11 chain and was identified with rat anti-mouse Vβ11-PE (BD 553198) or ASNENMDAM/H-2Db Pentamer (Proimmune F009-2A-G).

Adoptively transferred murine T cells were also examined using, rat anti-mouse thy1.1-PECy7 (eBioscience 25-0900-82), rat anti-mouse CD44-V450 (BD 560451) and rat anti-mouse CD62L-APC antibodies (BD 553132). Human T cells were analysed using anti-human CD4-FITC (BD 555346), CD8α-PECy7 (BD 557746), CD8β-APC (BD 544058), rat anti-mouse TCRβ-PE (BD 553172). Acquired FACS data was exported as FCS files and analysed using FlowJo software version 7.6.5 (Treestar). The gating strategy was performed consistently by first gating on live lymphocytes followed gating on CD8α+ or CD4+ populations. As the fluorescence intensity was expressed on a log scale, the geometric MFI was measured using FlowJo software.

2.5 In-vitro functional assays

2.5.1 Peptides

The following synthetic MHCI restricted peptides were used: MDM100 (YAMIYRNL) of the MDM2 protein is presented by H2-Kb, NP366 (ASNENMDAM) of the Influenza-A virus nucleoprotein, and the control peptide SV9 (FAPGNYPAL) derived from the Sendai virus are both presented by H2-Db. Peptides were reconstituted in PBS to a concentration of 2 mM and stored at −20°C. All peptides were synthesised by ProImmune (Oxford, UK).

2.5.2 Peptide loading of RMAS and T2 target cells

The standard concentration of saturating peptide used for all functional assays was 10 µM. To perform peptide titration experiments, the standard peptide concentration (10 µM) was first constituted followed by ten-fold dilutions (1 µM, 100 nM, 10 nM, 1 nM, 100 pM, 10 pM). 1 $x10^6$ temperature-induced RMAS cells or T2 cells were incubated for 2 hour in 500 µl of RPMI medium with each cognate peptide concentration (or control peptide at 10 µM). The target cells were irradiated with 80 Gy to stop proliferation before incubation with transduced T cells.

2.5.3 IFN-γ and lL-2 ELISA assay

TCR-td T cells (1-5 x 10^3) were incubated with peptide loaded irradiated RMAS cells (1 x 10^4) or EL4 cells (1 x 10^4) in triplicate in 96 well plates (TPP 92097) and kept humidified at 37°C and 5% CO_2 for 16 hours. The next day, either 50 µl of supernatant was harvested and tested in the IFN-γ or IL-2 ELISA assay or the plate was frozen for future analysis.

Cytokines IFN-γ and IL-2 of both murine and human origins were measured in the culture supernatant of TCR-td T cells using protocols derived from BD ELISA kits (cat no. 555138, 555148, 555142, 555190). 96-well Microtest ELISA plates (BD Falcon 353279) were coated overnight at 4°C with 50 µl per well with rat anti-mouse IFN-γ or IL-2 capture antibody at 2 µg/ml in coating buffer at pH 9.5. ELISA reagents were taken from a set kit (BD OptEIA Reagent Set B 550534) and the assay was performed at room temperature (RT). Plates were washed five times with wash buffer and then blocked with 200 µl per well of assay diluent for 1 hour. Plates were then washed a further five times with wash buffer. Recombinant mouse and human IFN-γ or IL-2 was prepared in assay diluent as standards. The maximum standard for the different cytokines were as follows: 2000 ng/ml for murine IFN-γ, 200 ng/ml for murine IL-2, 300 ng/ml for human IFN-γ and 500 ng/ml for human IL-2. Serial 1:2 dilutions from the maximum standard were performed six times and the assay diluent was used as the zero concentration standards. 50 µl of standard or culture supernatant sample was added to each well and incubated for two hours. Plates were then washed five times with wash buffer and 50 µl per well of biotinylated anti-IFN-γ or anti-IL-2 antibody diluted in assay diluent was added at 2 µg/ml. Plates were incubated for one hour and washed five times. 50 µl of streptavidin-horserasdish peroxidase was added to each well at RT for an hour and washed eight times. 50 µl of TMB substrate solution was next added and plates were incubated in the dark for 30 min to allow the colour to develop. At the end of incubation 50 µl per well of 0.25M sulphuric acid was added. The OD results from individual wells were read with a plate reader at 450 nm wave length. The standard curve is drawn using an Excel spreadsheet and the converted results was transferred onto Prism5 (Graphpad) to

generate graph and statistical analysis. Data are presented as the mean IFN-γ concentration (ng/ml) ± standard deviation of triplicate values.

2.5.4 IFN-γ and IL-2 intracellular cytokine assay

TCR-td T cells (1-5 x 10^3) were mixed with peptide loaded EL4NP or EL4 cells (1 x 10^4) in triplicate along with controls: T cells stimulated with 50ng/ml PMA (Sigma P1585) and ionomycin (500 mg/ml) (Sigma-Aldrich I9657) or T cells alone. After 1 hour, Brefeldin-A (1 µg/ml) was added to every well and the cells were maintained at humidified at 37°C and 5% CO_2 for 6 hours.

At the end of the incubation period, the cells were stained for CD4, CD8α and CD8β then fixed and permeabilised using reagents from a kit (BD 554714) and stained for intracellular cytokines using anti-IL-2 APC (BD 554429) antibodies and anti-IFN-γ PE antibodies (BD 554412). The cells were washed using Fix and Perm wash and FACS analysis was performed.

2.5.5 Cytotoxicity assay

The ability of F5-TCR-td CD4+ T cells to kill EL4 tumour cells (Chapter 5) was determined using a standard 4-hour ^{51}chromium (^{51}Cr)-release assay, which was performed on day 5 after transduction. EL4NP or EL4 (control) cells were then labeled with 37 MBq ^{51}Cr (Perkin Elma) for one hour at 37°C. ^{51}Cr-labelled target cells were then plated out in 100 µl at 5 x 10^3 cells per well in a round bottom 96-well plate. TCR-td cells were then added in 100 µl to achieve a starting effector to target ratio (E:T) of 50:1. Three additional T cell doubling dilution conditions were performed (25:1, 12:1, and 6:1). Spontaneous and maximal lysis was assessed by adding 100µl of media or 100µl 1% trifluoroacetic acid (TFA) (Sigma Aldrich 76-05-1) respectively to 5 x 10^3 ^{51}Cr-labelled target cells. Cells were incubated for 4 hours in a 37°C humidified incubator with 5% CO_2, after which time 100 µl of supernatant was collected. Samples were assessed for ^{51}Cr-release using a gamma-counter. Specific killing was calculated as follows:

$$\% \text{ specific killing} = \frac{(\text{experimental } ^{51}\text{Cr-release} - \text{spontaneous } ^{51}\text{Cr-release})}{\text{maximum } ^{51}\text{Cr-release} - \text{spontaneous } ^{51}\text{Cr-release}}$$

2.6 In-vivo studies

All procedures were carried out in accordance with UK Home Office regulations under an approved Project License.

2.6.1 Mouse lines

Female C57BL/6 thy1.1 (homozygous) mice were bred in house. Female C57BL/6 thy1.2 mice of the same age (three months) were bought from Harlan, UK.

Splenocytes were harvested from female C57BL/6 thy1.1 (homozygous) mice and used for T cell transduction. The donor mice were typed to confirm thy1.1 expression by analysing lymphocytes by tail bleed for presence of thy1.1 and absence of thy1.2. Prior to transduction and adoptive transfer the splenocytes were enriched for CD4+ T cells as previously described in section 2.3.2. The donor TCR-td T cells were predominantly CD4+ T cells (>95%) and the CD8+ T cells make up less than 0.5% of total lymphocyte population.

Female C57BL/6 thy 1.2 mice of the same age (4-6) months were used as recipient mice. Before irradiation, all mice were given Enrofloxacin (Bayer) in water and the right flank tumour injection site was shaved.

2.6.2 Bioluminescence

Growth of subcutaneously (sc) injected EL4NP tumour cells was monitored using bioluminescence at various time points. The EL4NP tumour cells were transfected with luciferase plasmid containing a CD34 marker. The EL4NP tumour cells were >80% CD34+ before injection.

To monitor bioluminescence in mice bearing EL4NPluc tumour cells, mice were anaesthetized and injected intraperitoneally with D-Luciferin firefly (Biosynth) at 7.5 mg/kg. Six minutes after injection, anaesthetized mice were imaged by Xenogen IVIS-100 (Caliper Life Sciences) using standard acquisition settings (f1.2, 10 seconds). The acquired images were analysed with living image 3.2 (Caliper Life Sciences) and graphs generated using Graphpad5 (Prism).

2.6.3 Tumour protection experiment

2.6.3.1 Tumour challenge and T cell transfer

Recipient female C57BL/6 mice were irradiated with 5.5 Gy at day 0, 6 hours before subcutaneous injection with 50 µl of 1 x 10^6 EL4NPluc cells in matrigel matrix (BD 354234) and PBS on the left flank. After injection, mice were imaged for presence of bioilluminescence and mice with no signal were re-injected. The next day (Day 1), mice were injected with TCR-td CD4+ T cells via the tail vein.

2.6.3.2 Monitoring tumour growth

Tumours were measured with calipers at different intervals and the growth evaluated by applying the formula (a^2 x b/2), where a=horizontal diameter and b=vertical diameter of the tumour. Mice were culled when tumour diameter exceeds 16mm or when ulceration occurs. Mice showing distress were culled in line with our UK Home Office project license which states that mice with weight loss greater than 20% or weight gain of more than 20% of the expected body weight, and mice with signs of

health deterioration or respiratory distress were killed according to a distress scoring system (Table X). Human endpoints stated by NCRI guidelines (Workman et al. 2010) were complied with throughout this project.

Animal identification	**Score**
Appearance	
Normal	0
Lack of grooming	1
Coat staring/ Piloerection, hunched up	2
Food and water intake	
Normal	0
Body weight down<5%	1
Body weight down <5-10%	2
Body weight down >15%	3
Natural behaviour	
Normal	0
Minor changes	1
Less mobile and alert, isolated	2
Self-mutilation, cachexia	3
Provoked behaviour	
Normal	0
Minor depression	1
Less mobile	2
Very weak and precomatose	3

Table 2-1 Distress scoring system used to determine end point. Mice is culled if total score >8, or scoring a 3 in any category, or any losing >20% of body weight, or tumour >16mm.

2.6.3.3 Monitoring of transferred CD4+ T cells

Blood sampling was performed on mice at Day 14-18 by removing 100-200 µl of blood by tail bleeding onto microvette containing Lithium heparin (Sarstedt 16.443).

To prepare blood samples for flow cytometry, each sample was exposed to 500 µl of dH_2O for 5 seconds before addition of 4.5 ml of isotonic PBS. Cells were then stained for flow cytometry as described in Section 2.3.2.

Mice were culled and spleen (Spl), lymph nodes (LN) and bone marrow (BM) removed to identify the presence of transferred T cells. Harvested lymphoid organs were mashed with RPMI medium and passed through a cell strainer (BD Falcon 352340). The lymphocytes were counted and made-up to 1 $x10^6$ cells/100 µl prior to staining for flow cytometry as described in Section 2.3.2. Total transferred T cells can be calculated by the multiplying the proportion of thy1.1+ CD4+ T cells with the total cell count.

2.6.4 Antigen re-challenge

Mice which became tumour free for >90 days were re-challenged with irradiated 1 $x10^6$ EL4NPluc tumour cells which was injected subcutaneously with PBS/Matrigel matrix (BD 354234) into the right lower limb. Seven days after re-challenge, mice were culled for analysis of TCR-td CD4+ T cells in draining and non-draining lymph nodes in the inguinal and popliteal regions. The preparation of the lymph nodes was as described in section 2.6.3.

Chapter 3

Chapter 3. Molecular biology

This chapter describes the molecular cloning performed to produce original vector constructs and the optimization of these constructs for transduction. The molecular and transduction techniques used were detailed in Section 2.1. The relevant vectors used in this project contain TCR and/or CD8 co-receptor transgenes.

3.1 TCR vectors

Some of the vector constructs were already used routinely by members of the department. These include the TCR constructs MDM-TCR (Bendle et al. 2007), F5-TCR (Morris et al. 2005) and CMV-TCR-CD8 constructs (Xue et al. 2013), all in the pMP71 vector backbone (Engels et al. 2003). The TCR vectors had been modified for enhanced expression by codon optimization, the introduction of additional cysteine residues, usage of picornavirus-derived 2A linker peptides between gene vectors and murine constant regions as detailed in Section 1.6.2. A summary of the TCR used is shown in table 3-1.

The MDM-TCR is in a bi-cistronic pMP71 vector with the TCRα and TCRβ chain separated by 2A-peptide sequences. The base sequences had previously been codon optimized to enhance expression in murine cells and a c-myc tag was added after the leader sequence of the TCRα chain to enable identification of the MDM-TCR.

The F5-TCR is also in a bi-cistronic pMP71 vector backbone with the TCRα and TCRβ chain also separated by 2A-peptide sequences. The base sequences were codon optimized for enhanced expression in murine cells. The CMV-TCR-CD8 construct was in quad-cistronic pMP71 vector with TCRα, TCRβ, CD8α and CD8β chains separated by 2A-peptide sequences. The base sequences had previously been codon optimized for expression in human cells and the constant regions were cloned from murine TCR constant regions, which has been demonstrated to enhance expression of the TCR in human t cells following transduction.

TCR	MHCI	Peptide specificity	Cells transduced	Identification
MDM	H2-Kb	YAMIRYNL	Murine T cells	Vβ11
F5	H2-Db	ASNENMDAM	Murine T cells	Vβ7 or c-myc tag on TCRα chain
CMV	HLA-A2	NLVPMVATV	Human T cells	Murine TCR constant chain*

Table 3-1 Summary of characteristics of modified TCR constructs used.
*The CMV-TCR is a hybrid TCR containing murine TCR constant regions.

3.2 CD8 vectors

3.2.1 Cloning the CD8β gene into the pMP71 retroviral vector

Figure 3.1 shows a schematic diagram of the cloning process used to produce the CD8β-pMP71 vector, which was used to transduce CD8β-/- CD8α+ T cells. The CD8β gene transcript containing wild-type (WT) DNA sequence in the Bluescript SK vector was a kind gift from Dr R Zamoyska (University of Edinburgh), the cloning sites were SalI and HINDIII. The CD8β chain was isolated by digestion of CD8β-SK vector with SalI and HINDIII restriction enzymes followed by gel electrophoresis and extraction (method shown in section 2.1.6). New NotI and EcoRI restriction sites at the 5' and 3' ends of the CD8β chain construct by PCR amplification (Section 2.1.4) using specific primers. The CD8β chain construct was then ligated into the pMP71 retroviral vector (method shown in Section 2.1.7) to produce the CD8β-pMP71 vector (Fig 3-1).

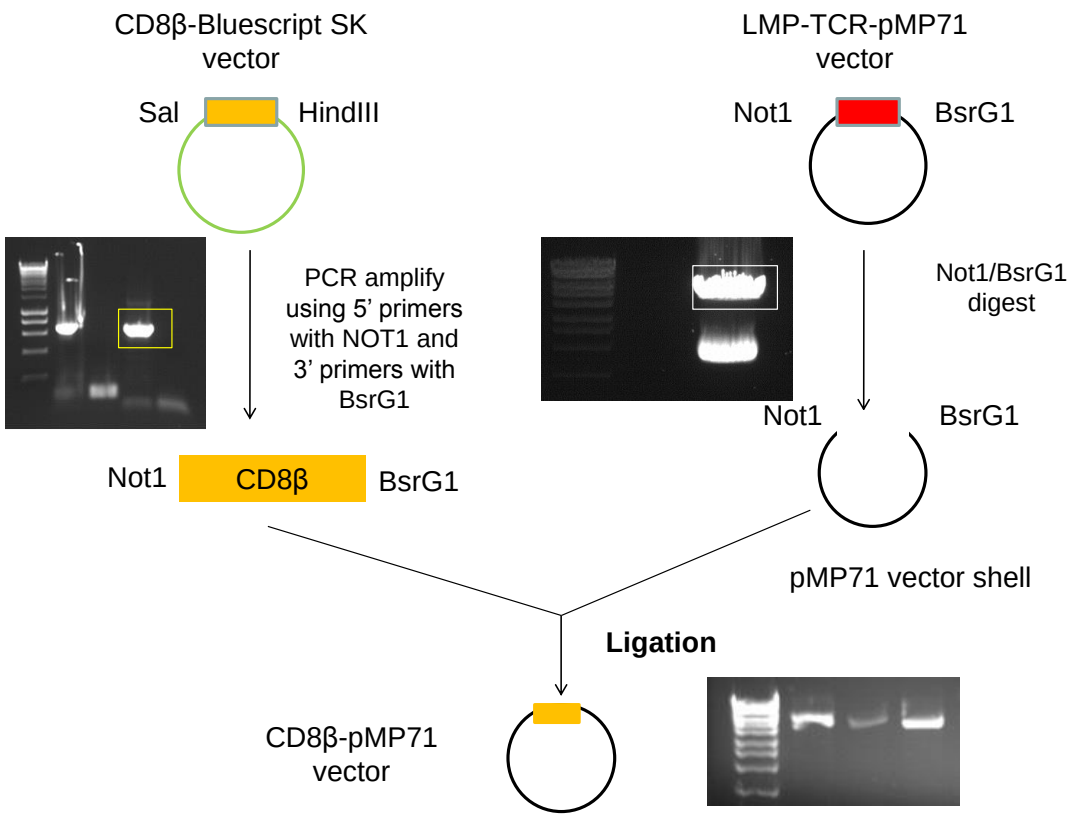


Figure 3-1 Derivation of CD8β-pMP71 vector.
The CD8β transgene (yellow) in Bluescript SK vector (green ring) was cloned into pMP71 (black ring) for the purposes of retroviral transduction of T cells.

3.2.2 CD8β gene mutagenesis

The CD8 co-receptor modification was focused on the CD8β chain due to its unique ability to enhance co-receptor function as highlighted in Section 1.4. The CD8β vector construct consists of a leader sequence and a coding sequence of 576 bp which encodes a 192 aa protein (Fig 3-2). Some CD8β chain mutations, in the immunoglobulin domain, have been shown to increase CD8 binding affinity with MHCI and enhanced the Ag-specific function of BW cell lines (Devine et al. 2006). These CD8β MHCI binding site mutants (S53L, S54V, L58R and L58R/I25A) were therefore candidate mutations for this project. The positions of the CD8β chain residues in the immunoglobulin-like domain that are involved in MHCI binding and that were targeted for mutagenesis (S53, S54 and L58) are shown in Fig 3-3. A second group of mutations involving O-glycosylation sites (T120A, T121A, T124A) were generated by the removal of threonine sites thus preventing O-glycosylation and sialylation. The position of these seven sites for mutagenesis is shown in upper case letters and coloured background in the CD8β gene sequence in (Fig 3-2).

```
  1 ctcattcagacccttcgtccctgctggttcaaaccaaccatacggcaaagatgtcctgt
  1  L  I  Q  T  P  S  S  L  L  V  Q  T  N  H  T  A  K  M  S  C

 61 gaggttaaaagcATCtctaagttaacaagcatctactggctgcgggagcgccaggacccc
 21  E  V  K  S  I  S  K  L  T  S  I  Y  W  L  R  E  R  Q  D  P

121 aaggacaagtactttgagttcctggcctcctggagtTCTTCCaaaggagttTTGtatggt
 41  K  D  K  Y  F  E  F  L  A  S  W  S  S  S  K  G  V  L  Y  G

181 gaaagtgtggacaagaaaagaaatataattcttgagtcttcagactcaagacggccctttt
 61  E  S  V  D  K  K  R  N  I  I  L  E  S  S  D  S  R  R  P  F

241 ctcagtatcatgaatgtgaagccagaggacagtgacttctacttctgcgcgacggttggg
 81  L  S  I  M  N  V  K  P  E  D  S  D  F  Y  F  C  A  T  V  G

301 agccccaagatggtctttgggacagggacgaagctgactgtggttgatgtccttcctACA
101  S  P  K  M  V  F  G  T  G  T  K  L  T  V  V  D  V  L  P  T

361 ACTgccccaACCaagaagactaccctgaagatgaagaagaagaagcaatgcccgttcccc
121  T  A  P  T  K  K  T  T  L  K  M  K  K  K  K  Q  C  P  F  P

421 cacccagagacccagaagggcctgacatgcagccttaccaccctcagcctgctggtagtc
141  H  P  E  T  Q  K  G  L  T  C  S  L  T  T  L  S  L  L  V  V

481 tgcatcctgcttctgctggcattcctcggagtggccgtctacttttactgtgtgcggagg
161  C  I  L  L  L  L  A  F  L  G  V  A  V  Y  F  Y  C  V  R  R

541 agagcccgaattcacttcatgaaacagtttcacaaa
181  R  A  R  I  H  F  M  K  Q  F  H  K
```

Figure 3-2 Mutagenesis of the murine CD8β gene.
The wild type CD8β gene sequence is shown here [base sequence (top row) and aa sequence (bottom row)]. Mutated positions where bases are indicated by coloured base code with capitalised alphabet.

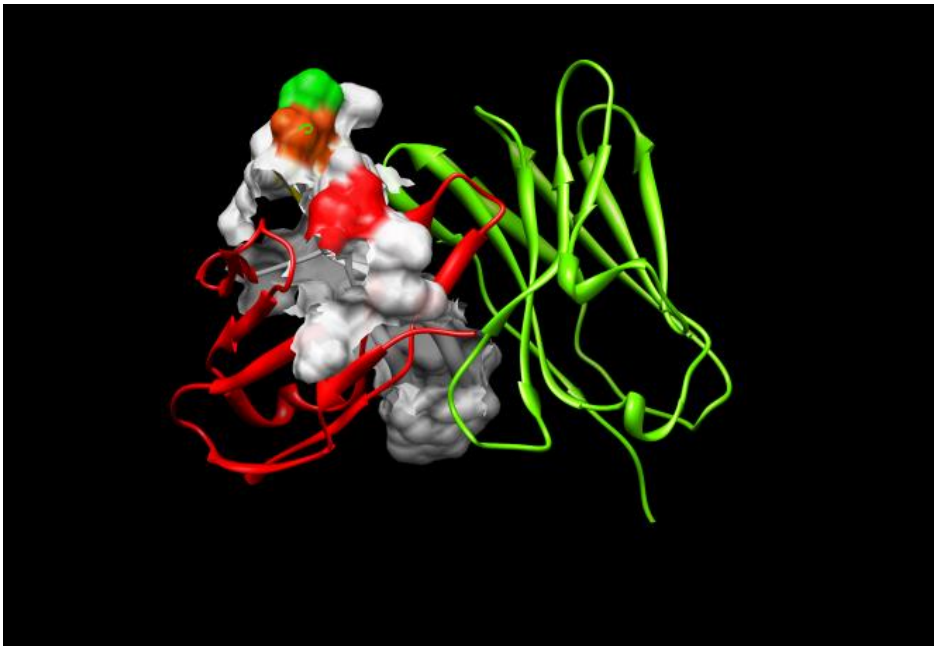


Figure 3-3 Graphic representation of the CD8 co-receptor showing the position of mutations known to enhance MHC-binding.
The CD8 co-receptor is shown as a ribbon/surface diagram based on crystallographic studies with the α-chain (green) and β-chain (red). The positions of mutations known to enhance MHC-binding are 53S (green), 54S (brown) and 58L (red).

Mutations were introduced within the murine CD8β chain transgene by site directed PCR mutagenesis (Section 2.1.5) using primers encoding the relevant base changes designed with Strategene primer design software (Table 3-2). A schematic diagram of this process for the MHC binding site mutants is shown in Fig 3-3 and for the glycosylation site mutants in Figure 3-4. Two of the desired CD8β sequences required the introduction of multiple mutations (L58R/I25A and T120A/T121A/T124A [TglyM]) and were produced by performing serial PCR mutagenesis. After PCR mutagenesis, the vectors were analysed by NotI and EcoRI digestion followed by gel electrophoresis (Fig 3-6) prior to sequencing to confirm the presence of the correct base mutations (Fig 3-7 and Fig 3-8).

CD8β mutants	
I25A	gatgtcctgtgaggttaaaagcgcctctaagttaacaagcatctac
L58R	ggagttcttccaaaggagttcggtatggtgaaagtgtggaca
S53L	gttcctggcctcctggagtcttccaaaggagttttgtat
S54V	cctggcctcctggagttctgtcaaaggagttttgtatggt
T120A	ggttgatgtccttcctgcaactgccccaaccaa
T121A	ttgatgtccttcctacagctgccccaaccaagaag
T124A	cttcctacaactgccccagccaagaagactaccc
T120A/T121A/T124A (TglyM)	tgatgtccttcctgcagctgccccaaccaagcagctgccccagccaagaagactaccc

Table 3-2 PAGE purified oligonucleotide primers.
Primers are designed using STRATAgene containing base changes (shown in red) used for PCR mutagenesis of CD8β (Anti-sense primers not shown).

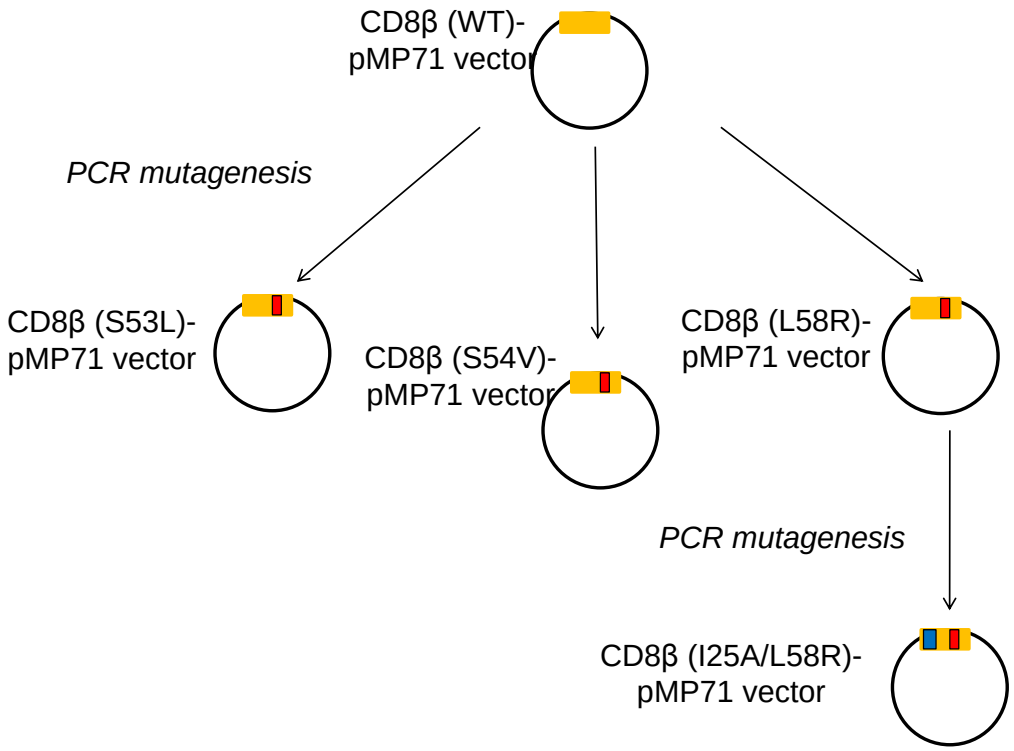


Figure 3-4 Introduction of CD8β MHC-binding site mutations by PCR mutagenesis.
The CD8β transgene is shown in yellow and mutagenesis in the transgene denoted by red (1st step) and blue (2nd step).

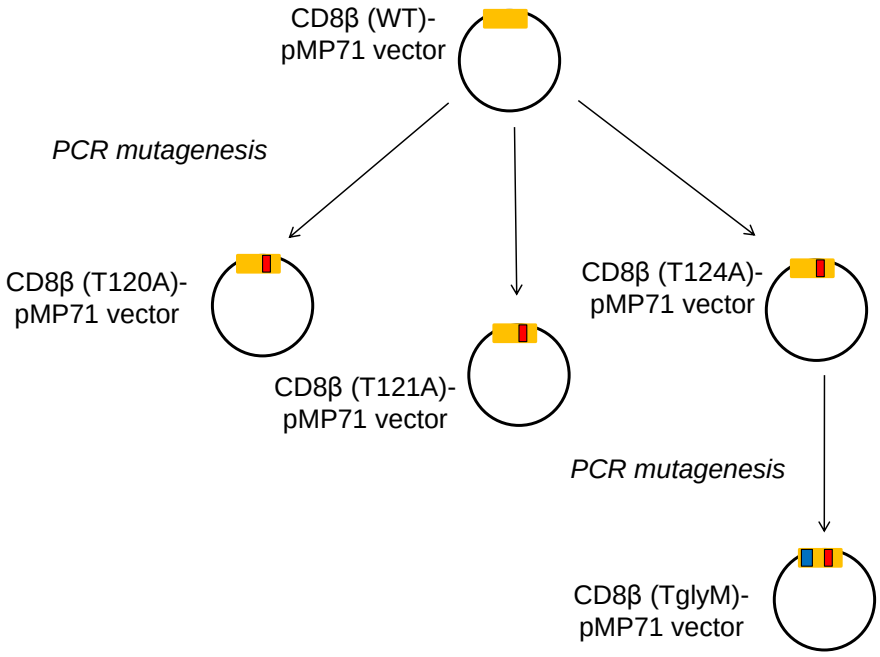


Figure 3-5 Introduction of CD8β- glycosylation site mutations by PCR mutagenesis. The CD8β transgene is shown in yellow and mutagenesis in the transgene denoted by red (1st step) and blue (2nd step).

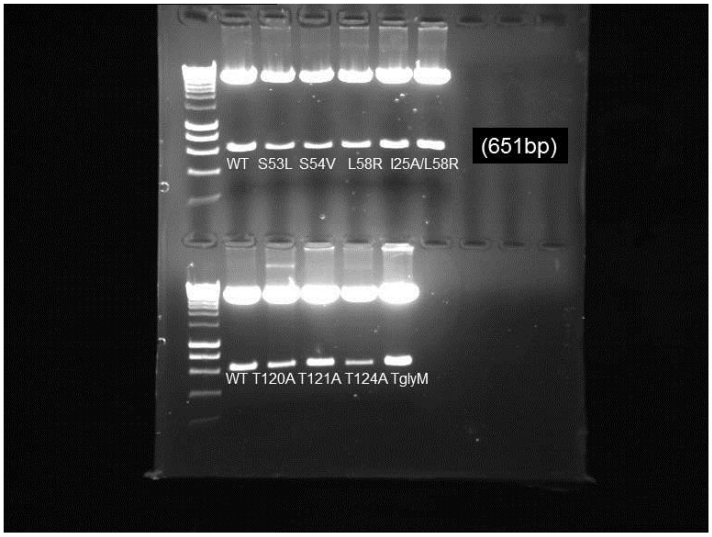


Figure 3-6 Agarose gel showing Not1 and BsrG1 digestion of all mini-prep DNA of E Coli colonies transformed using CD8β-pMP71 products

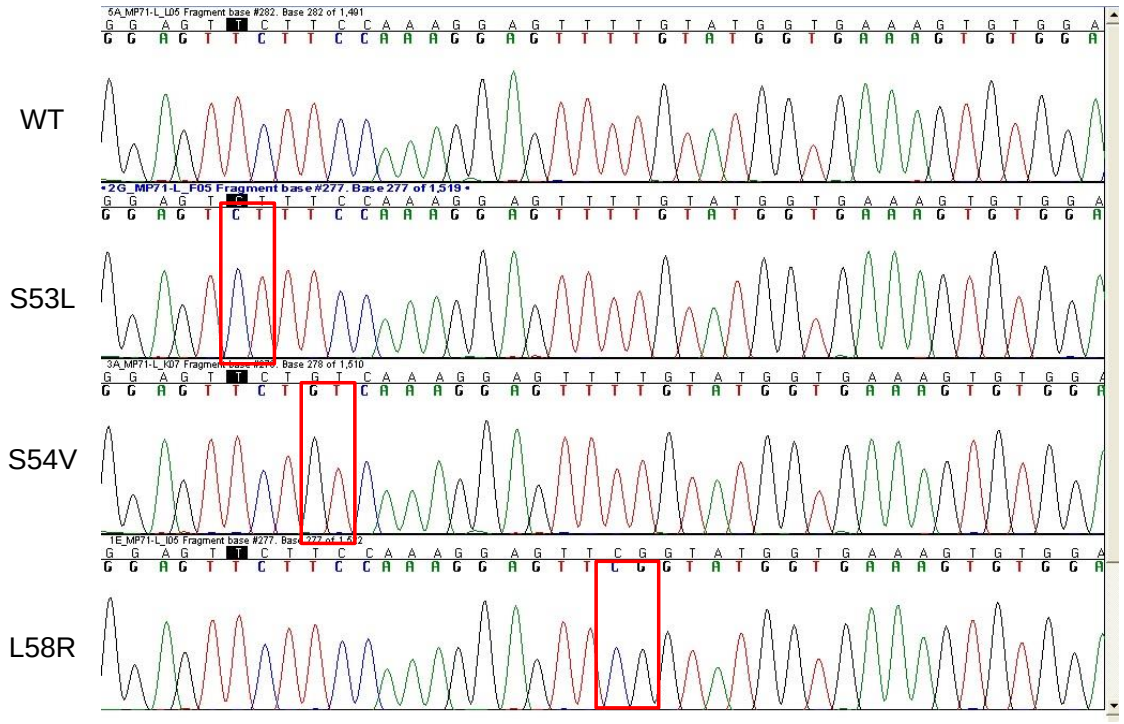


Figure 3-7 Sequence chromatograms confirming CD8β transgene base mutations. Mutations (highlighted in red rectangles) are introduced to the CD8β wild-type transgene (top row) to create CD8β MHC binding site mutants (S53L, S54V and L58R) using primers shown in Figure 3-2.

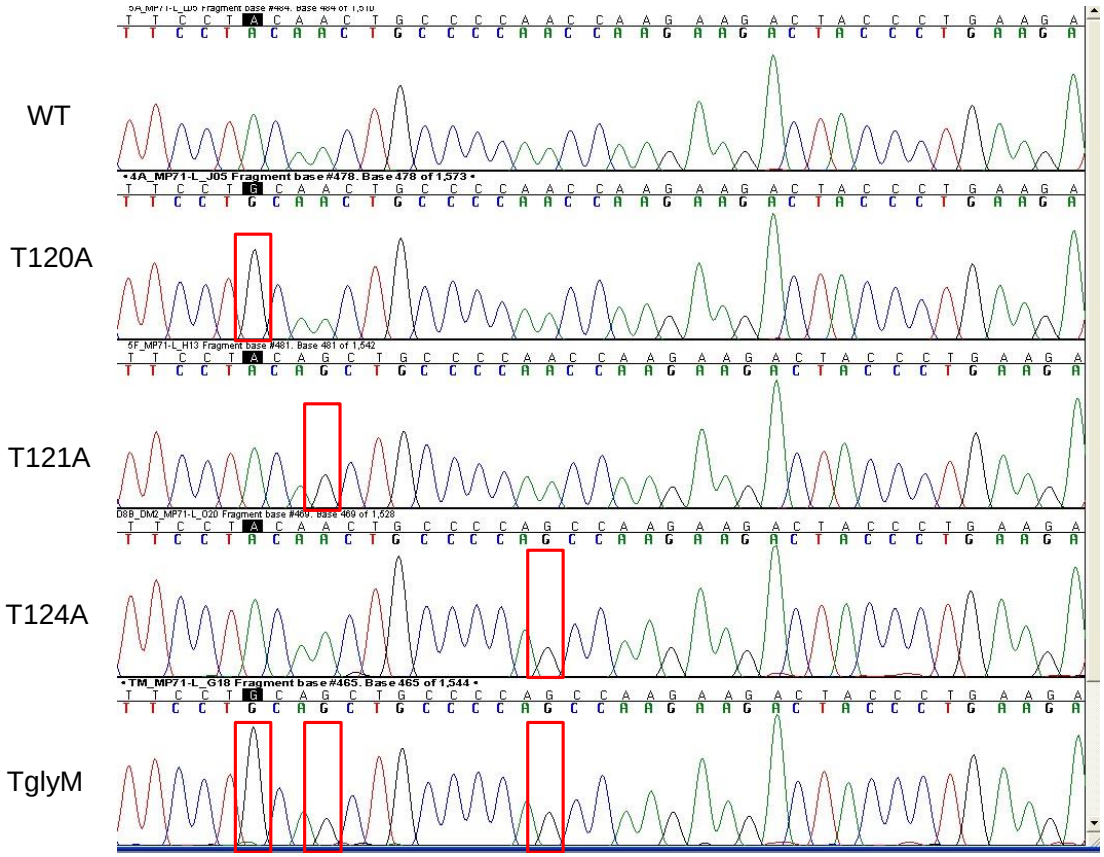


Figure 3-8 Sequence chromatograms confirming CD8β transgene base mutations. Mutations (highlighted in red rectangles) are introduced to the CD8β wild-type transgene (top row) to create CD8β glycosylation site mutants T120A, T121A, T124A and TglyM using primers shown in Figure 3-2.

3.3 Generation of retroviral vectors encoding both the TCR and CD8 co-receptor

At the start of the project the available F5-TCR and CD8 transgenes were cloned into separate vectors. Moreover the CD8 containing vector, CD8α-IRES-CD8β, has the less efficient pMX backbone (see section 2.1.1), which had been used in a previous project supervised by Dr E Morris. Therefore to study the effect of modified CD8 co-receptors on Ag-specific responses, a single vector construct was generated combining all the transgenes of the F5-TCR (TCRα and TCRβ chains) and CD8 co-receptor (CD8α and CD8β chains) into the optimised pMP71 vector backbone.

3.3.1 Construction of the F5-TCR-CD8 pMP71 retroviral vector

The available F5-TCR pMP71 construct which had been optimized and validated in our laboratory was used. The wild-type murine CD8 transgene synthesized by GeneArt (Invitrogen) was designed to be inserted into the F5-TCR pMP71 vector. The diagram showing the cloning strategy used is shown in Fig 3-9 and Fig 3-10. The proposed construct was designed to contain part of the TCR-constant β chain (Cβ) with a restriction site XhoI in the 5' end followed by CD8β and CD8α coding sequences separated by picornavirus-derived 2A linker peptides (Figure 3-10) which undergoes self-cleavage during translation (Donnelly et al. 2001). As the XhoI sequence was not unique in the pMP71 vector, an intermediate step using the pGA4 cloning vector was required (Figure 3-9).

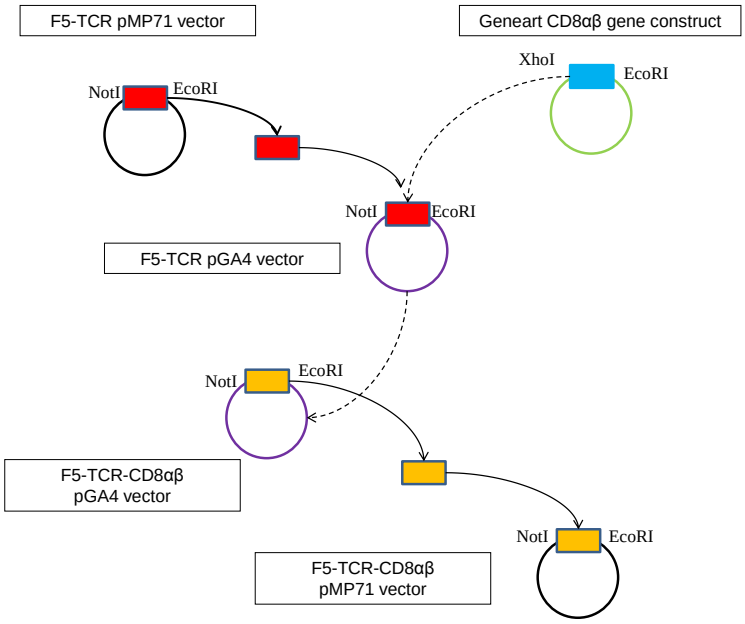


Figure 3-9 Schematic representation of the generation of the pMP71 vector containing F5-TCR, CD8α and CD8β coding sequences.
The F5-TCR transgene in pMP71 vector (red block in black circle) was combined with the CD8αβ transgene in pc3.1 vector (blue block in green circle) to form the large F5-TCR-CD8αβ transgene (yellow block) through an intermediary pGA4 vector (purple circle) before cloning into pMP71 vector (yellow block in black circle.

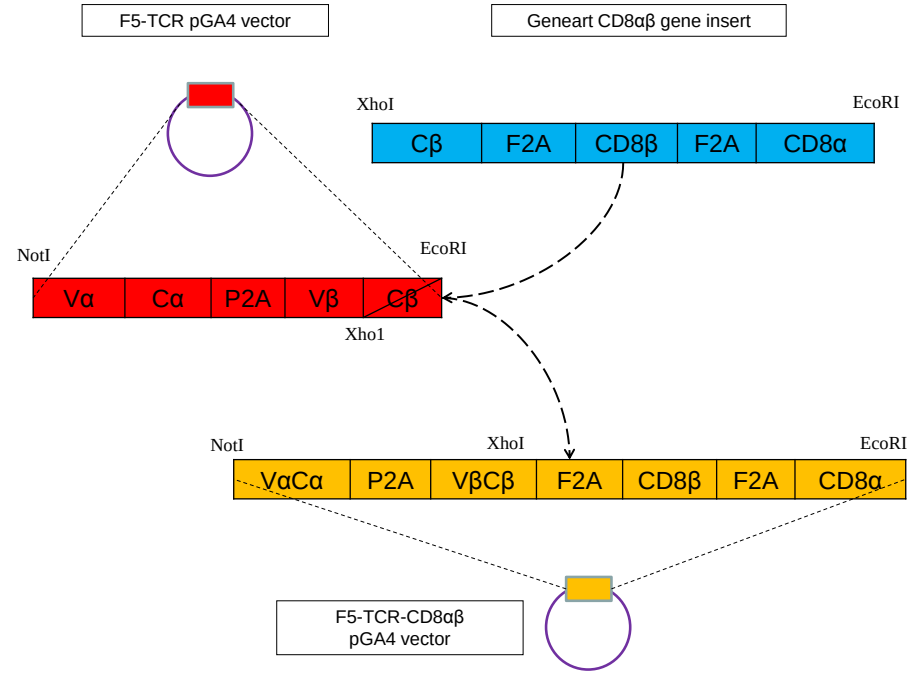


Figure 3-10 Summary of the molecular cloning required to derive the F5-TCR-CD8αβ pMP71 vector.
The combination of F5-TCR (red block) and CD8 transgene (blue block) was performed through overlapping of the TCR constant β chain (Cβ) present in both vectors and restriction sites XhoI and EcoRI in the pGA4 vector (purple ring).

3.3.2 Construction of the F5-TCR-CD8α and F5-TCR-CD8β pMP71 vectors

Variations of the F5-TCR-CD8 gene vector were made by the removal of either the CD8α or the CD8β transgenes through restriction site digestion followed by re-ligation of the vector. Removal of the CD8β transgene was designed to create the F5-TCR-CD8α vector, which could be used to transduce CD4+ T cells (Fig 3-11). Removal of CD8α transgene created the F5-TCR-CD8β vector for the transduction of CD8β-/- CD8α+ T cells (Fig 3-12). This strategy afforded flexibility to study the effects of F5-TCR-CD8α transduced CD4+ T cells as the CD8α expression may be important for memory responses (Madakamutil et al. 2004). The F5-TCR-CD8β vector was subsequently used to determine the efficacy of CD8β mutants in CD8+ T cells (Section 5.2).

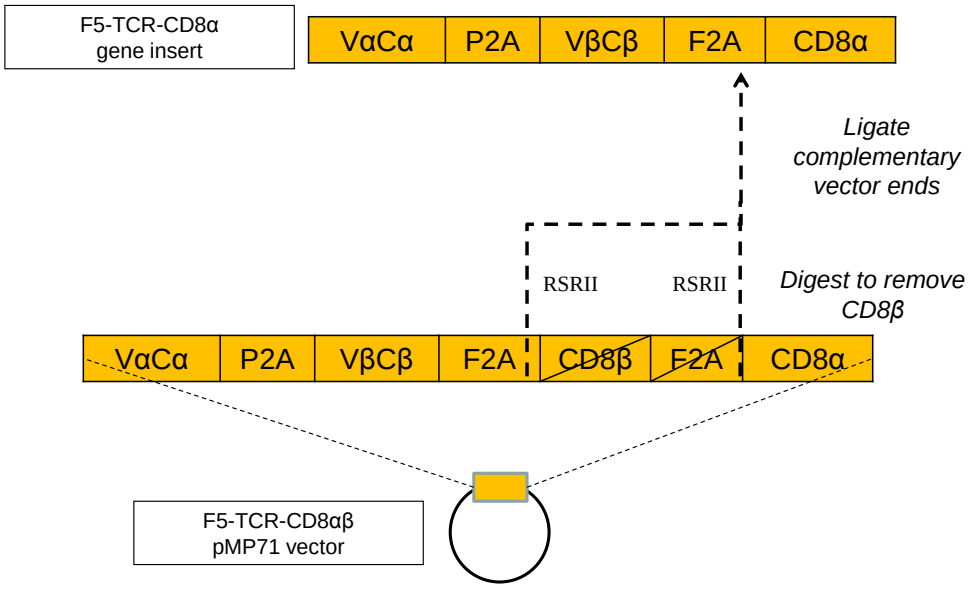


Figure 3-11 Schematic representation of the generation of the pMP71 vector encoding F5-TCR and CD8α coding sequences.
Digestion using restriction site RSRII removed the CD8β gene and the remaining vector ligated to form the F5-TCR and CD8α transgene.

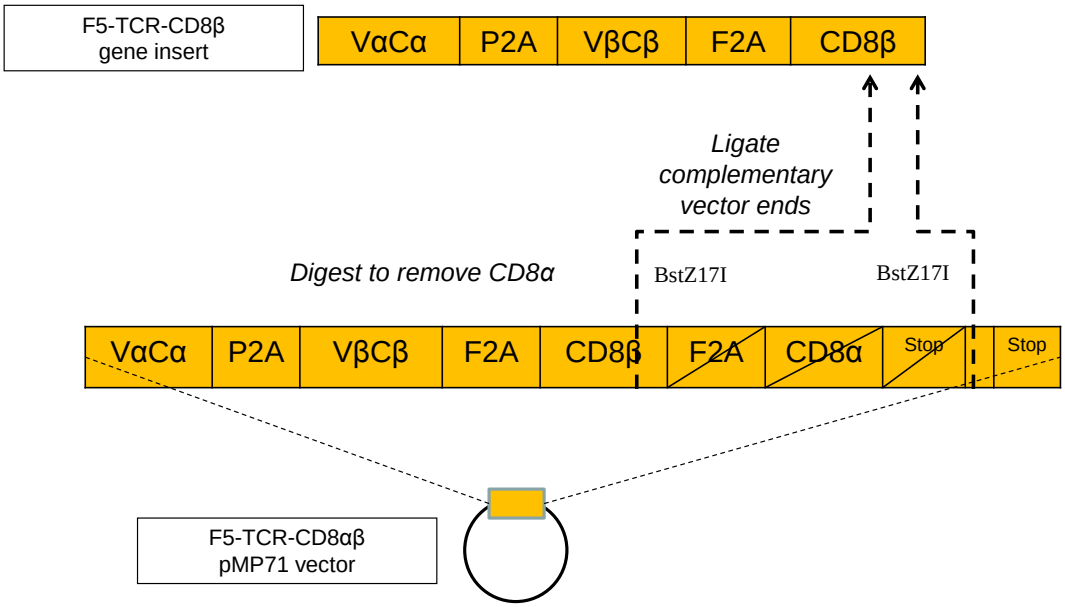


Figure 3-12 Schematic representation of the generation of the pMP71 vector containing F5-TCR and CD8β coding sequences.
Digestion using restriction site BstZ17I removed the CD8α gene and the remaining vector ligated to form the F5-TCR and CD8β transgene.

3.3.3 In vitro analysis of murine CD4+ T cells transduced with the F5-TCR-CD8 pMP71 vector

To test the F5-TCR-CD8 vectors, CD8neg CD4+ T cells taken from C57BL/6 mice were transduced with F5-TCR, F5-TCR-CD8α or F5-TCR-CD8αβ vectors. The FACS analysis (Fig 3-13) showed that although there was an increase in Vβ11 expression in all transduced populations compared to the negative control (CD4 unmodified), there was no detectable expression of either the CD8α or CD8β molecules. To determine why transduction with the above vectors failed to efficiently transfer the CD8 molecules the F5-TCR-CD8β vector was used to transduce CD8β-/- CD8α+ T cells taken from CD8β-deficient C57BL/6 mice with similar controls as above. FACS analysis of the transduced CD8β-/- T cells demonstrated CD8β successful expression (Fig 3-14).

The F5-TCR-CD8β transduced T cells were able to recognise NP peptide loaded RMAS or EL4-NP target cells and were of higher avidity than F5-TCR transduced CD8β-/- T cells (Fig 3-15), despite similar levels of F5-TCR expression as measured by Vβ11+ MFI (132 vs 112), as shown in Figure 3-14.

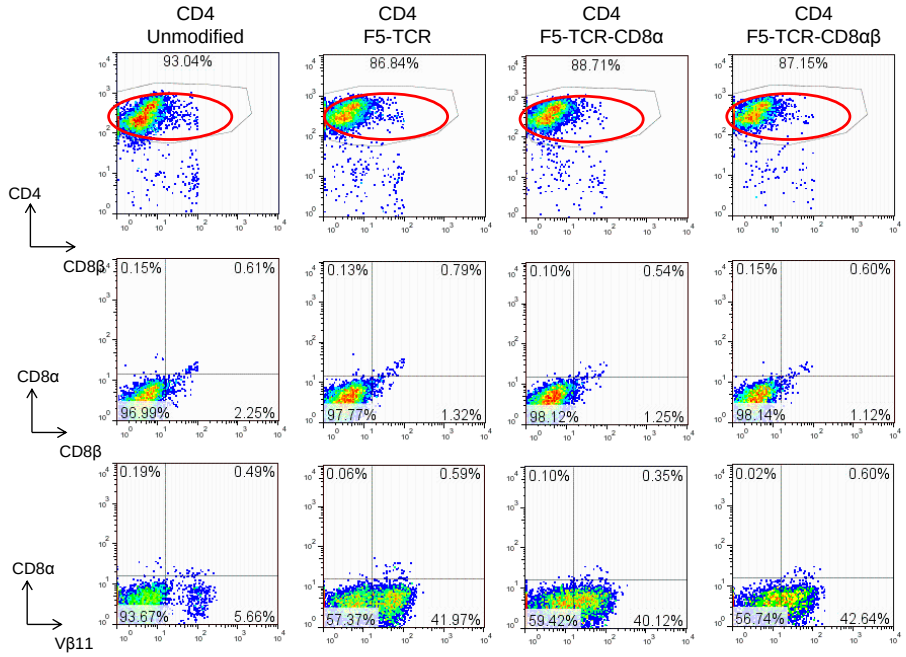


Figure 3-13 FACS plots showing transduction of CD4+ T cells using F5-TCR, F5-TCR-CD8α and F5-TCR-CD8αβ vectors.
Mock transduced CD4 T cells (unmodified) were used as control for gating purposes. The first row show that the sorted live lymphocytes were predominantly CD4+ T cells which were gated (red circle) and examined for expression of CD8 and TCR (Vβ11) on subsequent rows. Each column represents a separate condition.

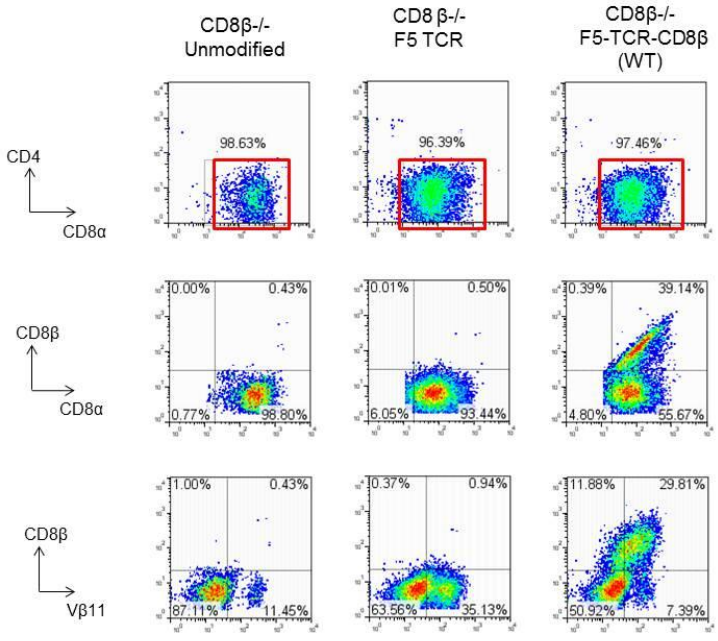


Figure 3-14 FACS plots showing transduction of CD8β-/- CD8+ T cells using pMP71 vectors containing the F5-TCR or F5-TCR-CD8β vectors.
Mock transduced CD8β-/- CD8+ T cells (unmodified) were used as control for gating purposes. The first row show that the sorted live lymphocytes were predominantly CD8α+ T cells which were gated (red square) and examined for expression of CD8β and TCR (Vβ11) on subsequent rows. Each column represents a separate condition.

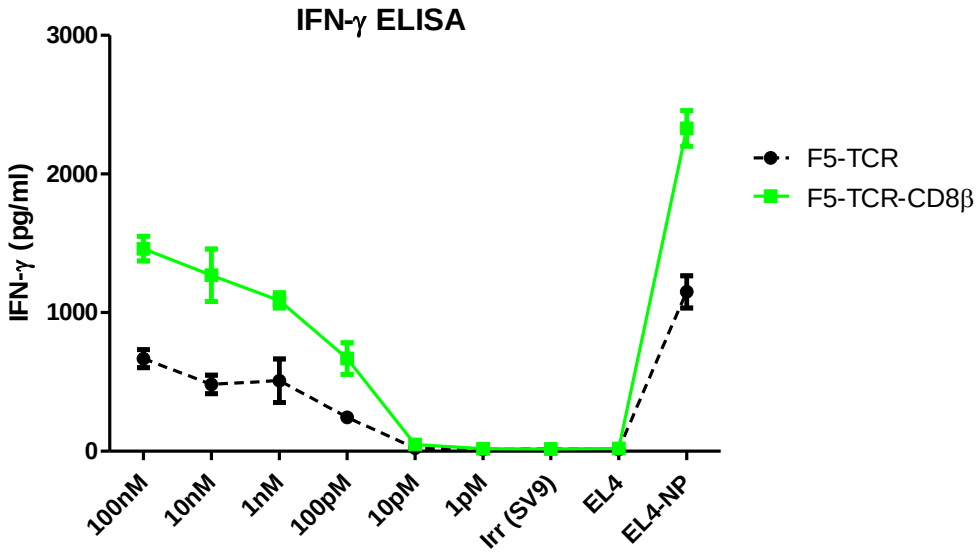


Figure 3-15 Enhanced Ag-specific IFN-γ secretion of CD8β-/- T cells transduced with the F5-TCR-CD8β (green line) retroviral vector compared to the F5-TCR vector (black line).
Transduced cells were incubated with RMAS loaded with different concentrations of relevant NP peptide or irrelevant (Irr) SV9 peptide. The transduced cells were also incubated with EL4 or EL4NP tumour cells. Supernatant was harvested 16 hours later and analysed for IFN-γ using ELISA.

3.3.4 Further development of the of F5-TCR-CD8 pMP71 vector

The F5-TCR-CD8αβ vector was revised due to null expression of both CD8α and CD8β. One obvious fault in vector design was related to the 2A peptide sequences. The two identical F2A peptide sequences were used in the GeneArt CD8 gene construct resulting in duplication of homologous regions, also known as direct repeats. The base sequence of GeneArt CD8 gene construct is shown in Appendix II. The presence of these repeats had been shown to cause deletion of the sequences in between the direct repeats (Julias et al. 1995). In the case of the F5-TCR-CD8 vector, the CD8β gene between the two F2A sequences may have been deleted (Fig 3-10). One of the F2A sequences was therefore replaced with a different 2A self-cleaving peptide (T2A).

The original vector contained a shorter version of the CD8α transgene (GenBank BC030679.1) because the longer CD8α transgene (GenBank: U34881.1) contained an untranslated region. However, in the revised vector, we reinstated this untranslated region in the CD8α transgene (Appendix III). The schematic diagram of the cloning process in which the revised T2A-CD8α transgene (synthesized by GeneArt, Appendix III) was inserted into the F5-TCR-CD8β vector is shown in Fig 3-16.

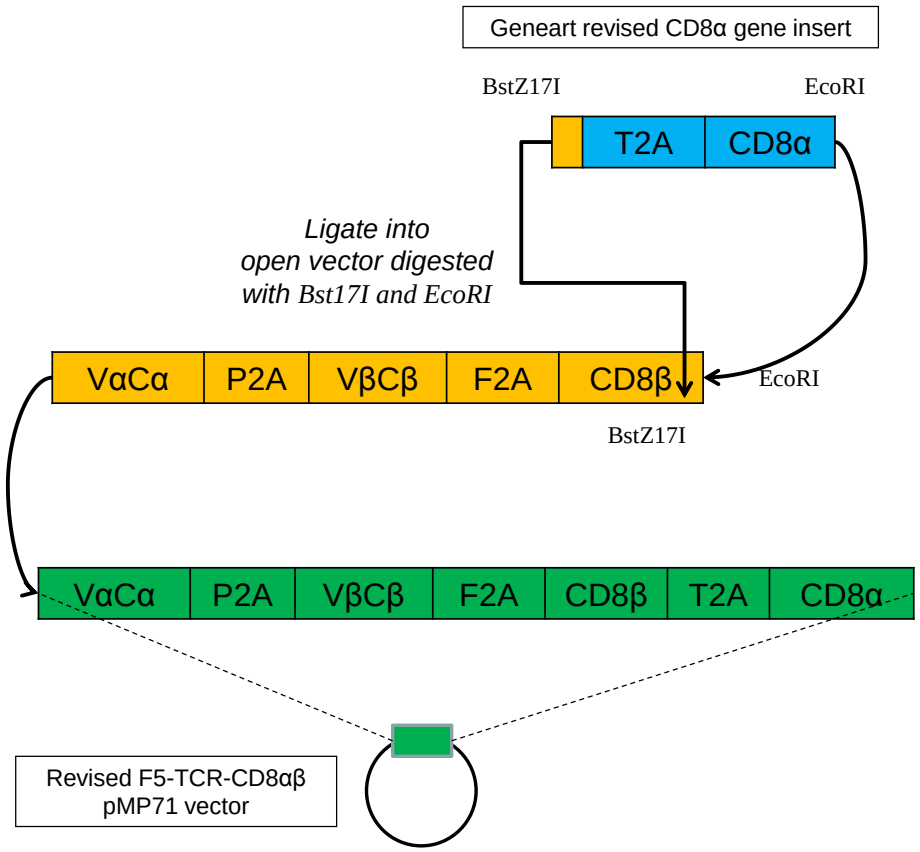


Figure 3-16 Schematic representation of the generation of the revised F5-TCR-CD8αβ pMP71 retroviral vector.
The original F5-CD8 transgene (yellow) was revised through removal of the F2A-CD8α transgene (cyan) using restriction sites BstZ17I and EcoRI and ligating with T2A-CD8α using the same restriction sites to produce the revised F5-CD8 transgene (green).

3.3.5 Transduction of murine CD4+ T cells with the revised F5-TCR-CD8 pMP71 retroviral vector

CD8neg CD4+ T cells were transduced with the F5-TCR, revised F5-TCR-CD8α or revised F5-TCR-CD8αβ vectors using our standard transduction protocols. Successful expression of TCR, CD8α and CD8β was demonstrated following transduction of CD4+ T cells (Fig 3-17). The increase in CD8α expression using the revised F5-TCR-CD8αβ and F5-TCR-CD8α vectors was 17% and 46% respectively compared to no CD8α expression (<0.5%) when the F5-TCR alone vector was used. The increase in CD8β expression using the revised F5-TCR-CD8αβ was 13.2% compared to no CD8β expression (<0.5%) when the F5-TCR vector was used. In the condition using the combined F5-TCR-CD8αβ vector expression of CD8α molecules was proportional to the CD8β molecules indicating heterodimer formation.

Subsequent FACS analysis demonstrated an increase in Vβ11 expression in all transduced cell populations compared to the untransduced cells, however the Vβ11+ percentage using the larger vectors F5-TCR-CD8αβ and F5-TCR-CD8α vectors were

considerably lower than the F5-TCR vector (6.72% and 22.65% vs 48.69%). More importantly the Vβ11+ MFI of the CD4+ T cells was inversely proportional to the size of the vectors used for transduction; F5-TCR-CD8αβ, F5-TCR-CD8α and F5-TCR vectors result in Vβ11 MFI: 55, 74 and 255 respectively.

Both the F5-TCR-CD8αβ and F5-TCR-CD8α transduced CD4+ T cells were able to recognise EL4-NP target cells and produce IL-2 in an Ag-specific manner (Fig 3-18). However when comparing the same number of Vβ11+ CD4+ T cells, the magnitude of IL-2 production of F5-TCR-CD8αβ and F5-TCR-CD8α were lower than F5-TCR transduced CD4+ T cells (Fig 3-18). This may be related to the lower F5-TCR expression as a result of using large F5-TCR-CD8 and F5-TCR-CD8α vectors to transduce CD4+ T cells.

The largest F5-TCR-CD8 vector consistently gave the lowest Vβ11 and CD8α expression as measured by MFI in primary CD4+ T cells. The low expression may be related to the size of the vector reducing the efficiency of protein production and expression. To reconfirm this observation, the F5-TCR-CD8 vectors were used to transduce TCR-negative CD8β-negative BW-cells and (Fig 3-19). Similar to the CD4+ T cells, the Vβ11+ MFI of the BW cells was inversely proportional to the size of the vectors used for transduction; F5-TCR-CD8αβ and F5-TCR vectors result in Vβ11 MFI of 342 and 536 respectively. Therefore despite the efforts put into producing the F5-TCR-CD8αβ gene construct, this was inferior compared to the F5-TCR alone vector and was not used further in this project.

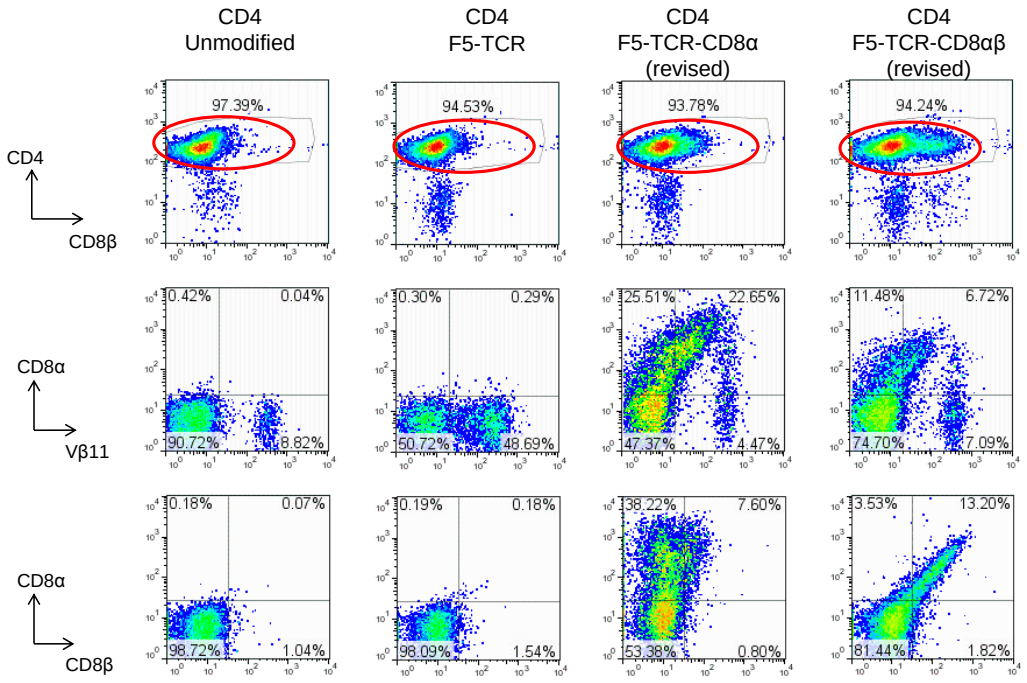


Figure 3-17 FACS plots showing transduction of CD4+ T cells using F5-TCR, F5-TCR-CD8α and F5-TCR-CD8αβ vectors.
Mock transduced CD4+ T cells (unmodified) were used as control for gating purposes. The first row show that the sorted live lymphocytes were predominantly CD4+ T cells which were gated (red circle) and examined for expression of CD8 and TCR (Vβ11) on subsequent rows. Each column represents a separate condition.

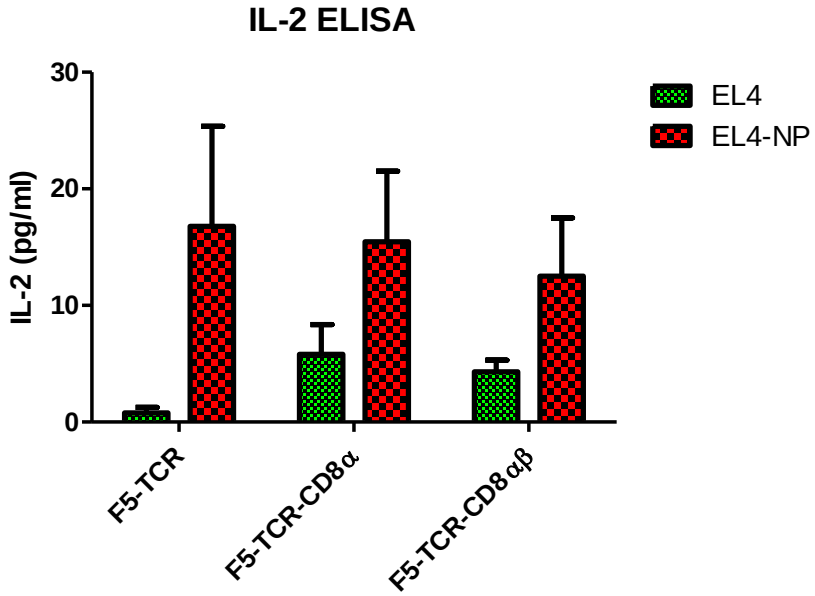


Figure 3-18 IL-2 ELISA assay comparing function of transduced CD4+ T cells.
CD4+ T cells are transduced with F5-TCR, F5-TCR-CD8α or F5-TCR-CD8αβ MP71 vectors and incubated EL4 tumour cells transfected with or without NP. Supernatant was harvested 16 hours later and analysed for IL-2 using ELISA.

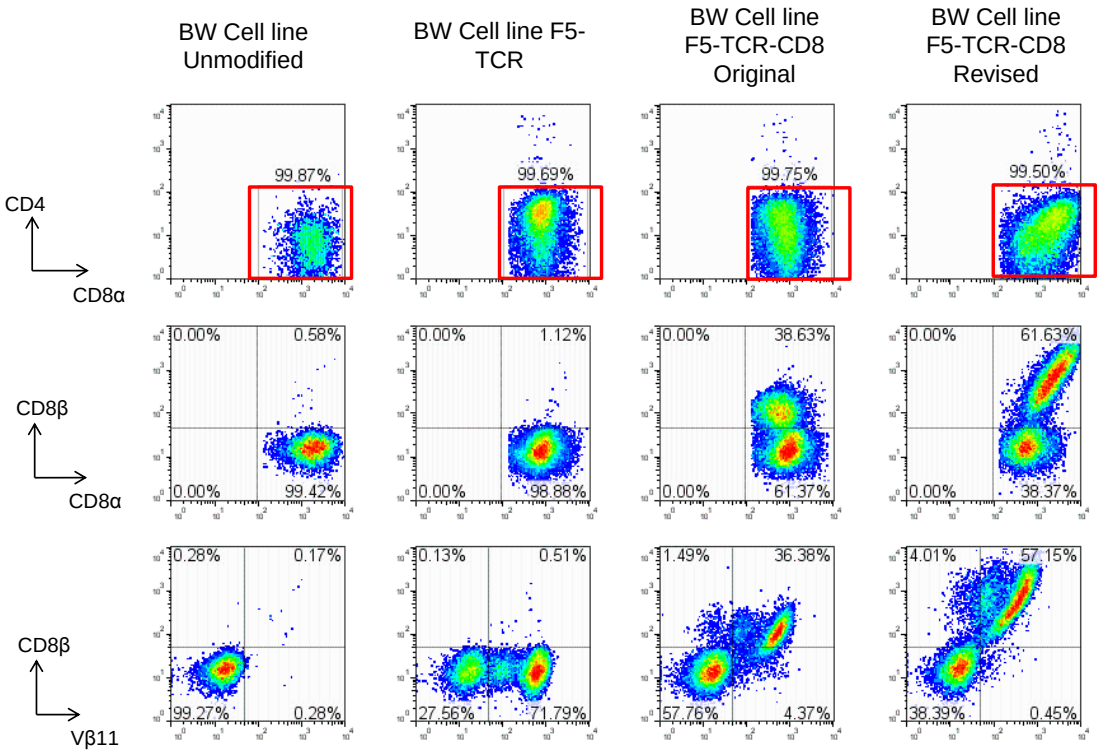


Figure 3-19 FACS plots showing transduction of BW cells using F5-TCR, F5-TCR-CD8αβ (original) or F5-TCR-CD8 (revised) vectors.
Mock transduced BW cells (unmodified) were used as control for gating purposes. The first row show that the sorted live BW cells were predominantly CD8α+ T cells which were gated (red square) and examined for expression of CD8β and TCR (Vβ11) on subsequent rows. Each column represents a separate condition.

3.4 Summary

Two murine TCR (MDM-TCR and F5-TCR) and one human TCR (CMV-TCR) were utilised in this project. All TCR constructs had been optimized for expression and were inserted into pMP71 retroviral vectors. The CD8β gene was inserted into pMP71 vectors before specific mutations were introduced by PCR mutagenesis and confirmed by sequencing. The CD8β mutants S53L, S54V, L58R, L58R/I25A, T120A, T121A, T124A and TglyM were created. A quad-cistronic vector combining F5-TCR and CD8 co-receptor transgene was created using molecular techniques. This large vector was less efficient for gene transfer with reduced F5-TCR expression compared to TCR alone vector following transduction, with a concomitant reduction in antigen specific functional responses. This was confirmed in both CD4+ T cells and BW cells.

Chapter 4

Chapter 4. In-vitro analysis in CD8β-/- T cells

4.1 Introduction

Chapters 2 and 3 described the generation of the retroviral viral constructs used in this project. As the F5-TCR-CD8 quad-cistronic vector was not functional, comparison of CD8 mutants was only possible using co-transduction of T cells with two separate vectors, a TCR vector (either the F5-TCR or the MDM-TCR) and the relevant CD8 vector.

CD8β mutants with enhanced MHCI binding have previously been reported by Devine et al and were shown to augment IL-2 production when introduced into a T-cell line. To investigate if similar effects could be observed in primary T cells, the 'augmenting' CD8β mutants were tested on CD8β-/- CD8+ T cells. The CD8β-/- T cells were derived from CD8β knockout mice obtained as a kind gift from Dr R Zamoyska (University of Edinburgh). Although these mice had five-fold less circulating mature CD8+ T cells (expressing the CD8αα-homodimer form of CD8 co-receptor) they were able to mount primary and secondary anti-viral responses (Angelov et al. 2009) and were not susceptible to opportunistic infections. Therefore the CD8β-/- CD8+ T cells were considered ideal for testing the impact of CD8β chain mutations. Eight candidate CD8β mutants were identified at the start of the project, which included four MHC-binding site mutants (S53L, S54V, L58R and L58R/I25A) and four O-glycosylation site mutants (T120A, T121A, T124A and T120A/T121A/T124A [TglyM]). The generation of the CD8β vectors containing the mutations was described in detail in Section 3.4.

The MDM(3f3b)-TCR used in this project was one of two MDM100/H2-Kb specific TCRs isolated and characterised by the laboratory (Sadovnikova & Stauss 1996). The MDM(3f3b)-TCR and the MDM(6a5d)-TCR both recognise the same MDM100 peptide derived from the murine double minute-2 (MDM2) protein, which is a TAA over expressed in many cancers such as lymphomas, sarcomas, breast and urothelial cancers. As the T cell clones containing the MDM(3f3b)-TCR were high avidity and CD8 co-receptor dependent (McNicol et al. 2007), this TCR was chosen to be tested with various CD8 co-receptor mutants. The MDM(6a5d)-TCR was of lower avidity and CD8 independent and was not used in this project. The MDM(3f3b)-TCR is codon optimised and contain a c-myc tag in the leader sequence of the TCR-α chain. Introduction of c-myc tag into this position in OT1-TCR, gp100-TCR and P14-TCR was shown not to alter TCR function (Kieback et al. 2008). The c-myc tag was introduced and tested in our laboratory by Dr Sara Ghorashian and was found to have similar

functional properties to the original MDM-TCR based on peptide titration cytokine release and cytotoxicity assays.

The second TCR examined was the F5-TCR which recognises the NP366 peptide derived from Influenza virus-A nucleoprotein. Tumour protection studies have been performed using both CD8+ and CD4+ T cells transduced to express the F5-TCR. Ag-specific IFN-γ production was used as the main functional read out for the TCR-td CD8+ T cells in this chapter.

4.2 Co-transduction of CD8β mutants and the MDM-TCR into CD8β-/- CD8+ T cells

4.2.1 Successful cell surface expression of the MDM-TCR and CD8β mutants following transduction of CD8β-/- CD8+ T cells

CD8β-/- CD8α+ T cells were transduced with pooled viral supernatant containing a 1:1 mixture of MDM-TCR and CD8β (wild-type WT, or mutated) vectors. The CD8β constructs used were either WT or one of four MHC-binding site mutants (S53L, S54V, L58R and L58R/I25A) or one of four O-glycosylation site mutants (T120A, T121A, T124A and T120A/T121A/T124A [TglyM]). Mock-transduced (unmodified) or MDM-TCR alone transduced CD8β-/- CD8α+ T cells were used as controls. Three days after transduction, T cells were analysed for surface expression of TCR and/or CD8β using fluorochrome-conjugated antibodies (Fig 4-1 to 4-3). As separate retroviral vectors were used for transduction, two distinct populations were seen: a single-transduced c-myc+ population and a double-trandsuced CD8β+ c-myc+ population. The proportion of single-transduced c-myc+ CD8+ T cells (ie., MDM-TCR expressing) in each condition was observed to be broadly equivalent at between 31.65% and 41.98% of total CD8+ T cells. The double-transduced CD8β+ c-myc+ population comprised between 1.94% and 10.96% of total CD8+ T cells in all conditions except the mock-transduced negative control and the MDM-TCR alone conditions, as expected. Interestingly, most CD8β+ transduced T cells were also c-myc+, indicating that successful expression of CD8β may have improved TCR-transduction or expression in CD8β-/- T cells. The expression of CD8β in the transduced populations was proportional to the endogenous CD8α expression. The expression level of transduced CD8β as measured by MFI was similar between WT (MFI: 237) and all mutations (MFI: 187 to 261) except S53L (MFI: 77). Even the CD8β mutation with three glycosylation sites mutated (TglyM) had good expression (MFI: 191). These expression features were consistently observed in at least three repeat transductions.

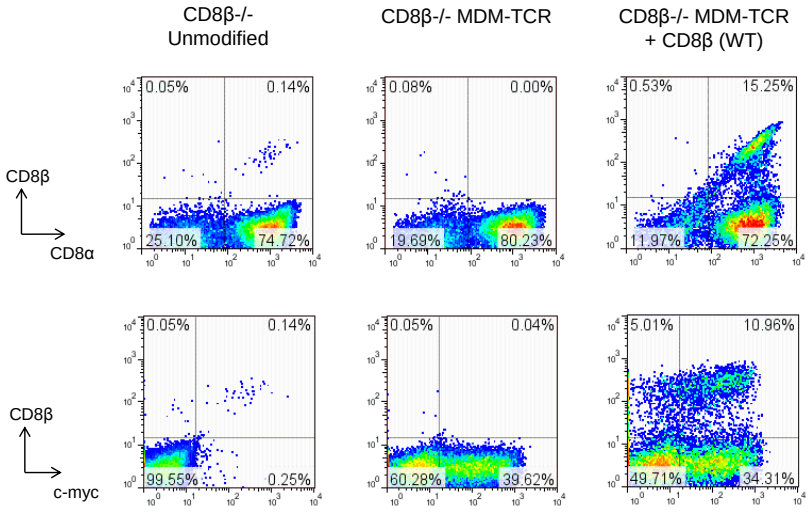


Figure 4-1 FACS analysis of CD8β-/- T cells transduced with retroviral vectors encoding the MDM-TCR and CD8β (WT) in separate vectors.
Mock transduced CD8β-/- T cells (unmodified) were used as control for gating purposes. The sorted live lymphocytes were predominantly CD8α+ T cells which were examined for expression of CD8β and TCR (c-myc). Each column represents a separate condition.

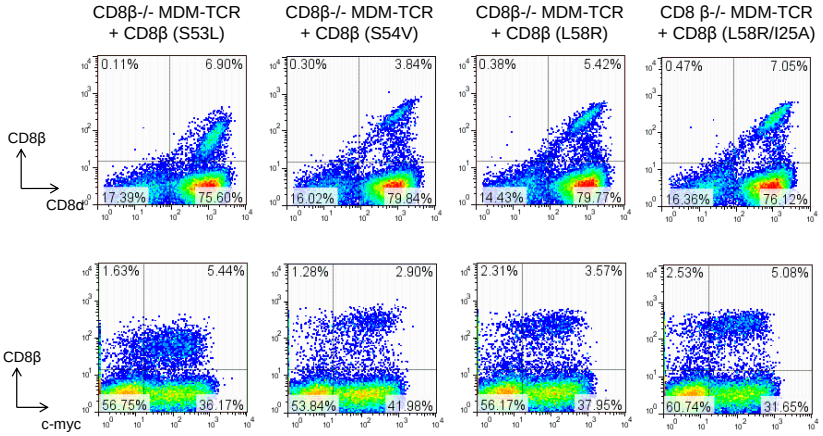


Figure 4-2 FACS analysis of CD8β-/- T cells transduced with the MDM-TCR and CD8β MHC-binding site mutants (S53L, S54V, L58R and L58R/I25A) in separate vectors.
Mock transduced CD8β-/- T cells (unmodified) were used as control for gating purposes. The sorted live lymphocytes were predominantly CD8α+ T cells which were examined for expression of CD8β and TCR (c-myc). Each column represents a separate condition.

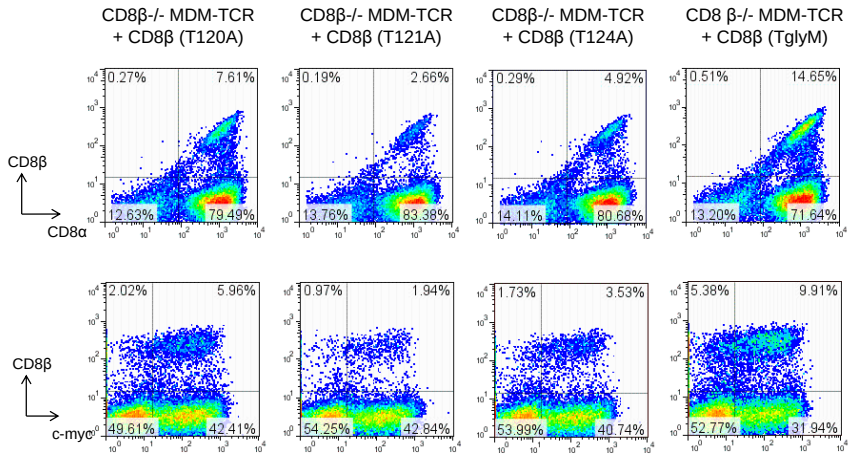


Figure 4-3 FACS analysis of CD8β-/- T cells transduced with the MDM-TCR and CD8β glycosylation mutants in separate vectors.
Mock transduced CD8β-/- T cells (unmodified) were used as control for gating purposes. The sorted live lymphocytes were predominantly CD8α+ T cells which were examined for expression of CD8β and TCR (c-myc). Each column represents a separate condition.

4.2.2 Antigen-specific IFN-γ production of MDM-TCR-td CD8β-/- T cells

As the MDM-TCR-td cells were known to be highly CD8 co-receptor dependent with respect to Ag-specific function (McNicol et al. 2007) and the proportion of single MDM-TCR-td cells were similar between different conditions, cell sorting was not performed to separate the single and double transduced populations prior to functional assays being performed. To compare the effect of expressing CD8β WT or CD8β mutants in TCR-td CD8β-/- T cells, the same number of double transduced CD8β+ and MDM-TCR + T cells from each condition (various CD8β constructs) were incubated with saturating peptide-loaded RMAS target cells (in triplicate). After overnight incubation, supernatant was removed and assessed for IFN-γ production by ELISA (Fig 4-4 and 4-5). Singly transduced MDM-TCR-td CD8β-/- T cells produced relatively small amounts of IFN-γ (<50 pg/ml) (Fig 4-4) in response to NP peptide loaded RMAS cells. However, the expression of CD8β WT or any CD8β mutant (except S53L) resulted in an increase in Ag-specific IFN-γ production (>100 pg/ml). Overall, the concentration of IFN-γ produced was low and background responses were high. Therefore comparisons made between the functional effect of expressing the various CD8β constructs were limited. Despite such constraints, the co-transfer of CD8β (L58R) and CD8β (L58R/I25A) co-receptors produced greater amounts of IFN-γ.

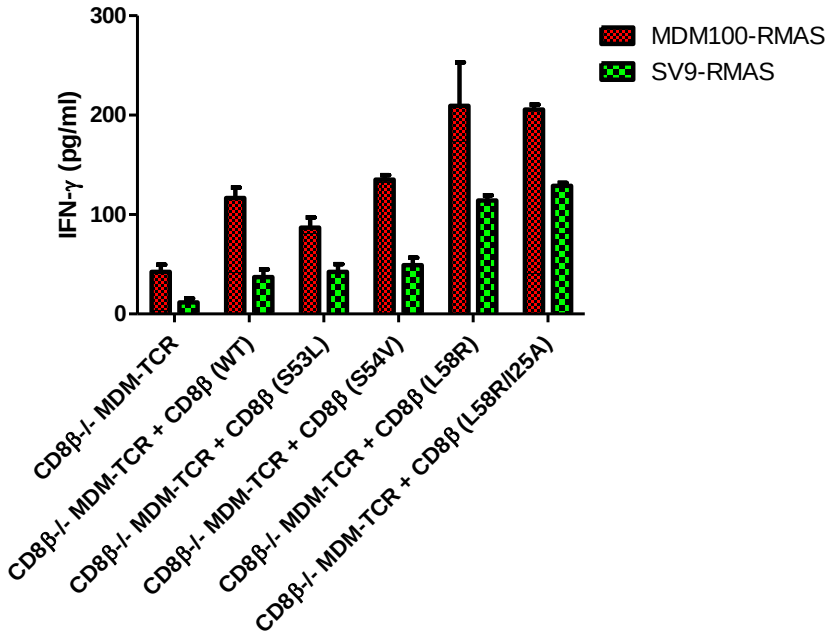


Figure 4-4 Recognition of RMAS cells loaded with saturating concentrations of peptide induces IFN-γ production by TCR transduced CD8α+ T cells.
ELISA assay using CD8β-/- CD8+ T cells transduced with MDM-TCR vector and CD8β vector containing wild-type (WT) or MHC binding site mutant (S53L, S54V, L58R or L58R/I25A) DNA sequences stimulated with RMAS cells loaded with saturating concentrations (10μM) of relevant MDM100 peptide or irrelevant SV9 peptide for 16 hours in triplicate. This is a representative result of at least two independent experiments.

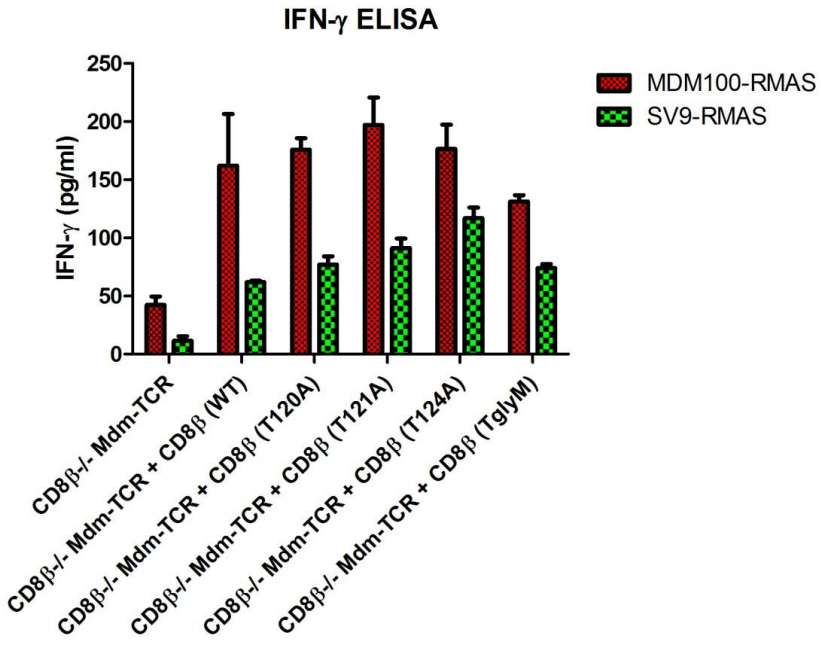


Figure 4-5 Recognition of RMAS cells loaded with saturating concentrations of peptide induces IL-2 production by TCR transduced CD8α+ T cells.
ELISA assay using CD8β-/- CD8+ T cells transduced with MDM-TCR vector and CD8β vector containing WT or glycosylation site mutant (T120A, T121A, T124A and TglyM) DNA sequences stimulated with RMA-S cells loaded with saturating concentrations (10μM) of relevant MDM peptide or irrelevant SV9 peptide for 16 hours in triplicate wells. This is a representative result of at least two independent experiments.

To show that the low levels of Ag-specific IFN-γ production were related to the MDM-TCR rather than the functional capacity of CD8β-/- CD8+ T cells, CD8+ T cells taken from wild-type C57BL/6 mice were transduced with the same MDM-TCR and the F5-TCR as a control. When the same number of TCR-td CD8+ T cells was incubated overnight with saturating peptide loaded RMAS target cells, the Ag-specific IFN-γ response of F5-TCR-td CD8+ T cells was four-fold that of MDM-TCR-td CD8+ T cells (Fig 4-6). In subsequent experiments with F5-TCR transduced CD8β-/- CD8 T cells, significant Ag-specific responses were observed (Section 4.4.2).

The dysfunction of MDM-TCR transduced C57Bl/6 CD8+ T cells was studied by Dr Sara Ghorasian who found that these transduced T cells were more activated in vitro (CD44/CD62L, blasted) and showed upregulation of exhaustion markers, including PD-1, LAG-3, 2B4. The reduced viability related to increased apoptosis was attributed to native MDM expression by activated C57Bl/6 T cells. MDM-TCR transduced Balb/c CD8+ T cells had lower levels of apoptotic markers, greater viability and Ag-specific cytokine production. Absence of fratricide was inferred because the fraction of apoptotic cells was not greater than the transduction efficiency. The dysfunction of MDM-TCR transduced C57Bl/6 CD8+ T cells was noted before the introduction of the c-myc tag by Dr Downing a previous PhD student in the laboratory.

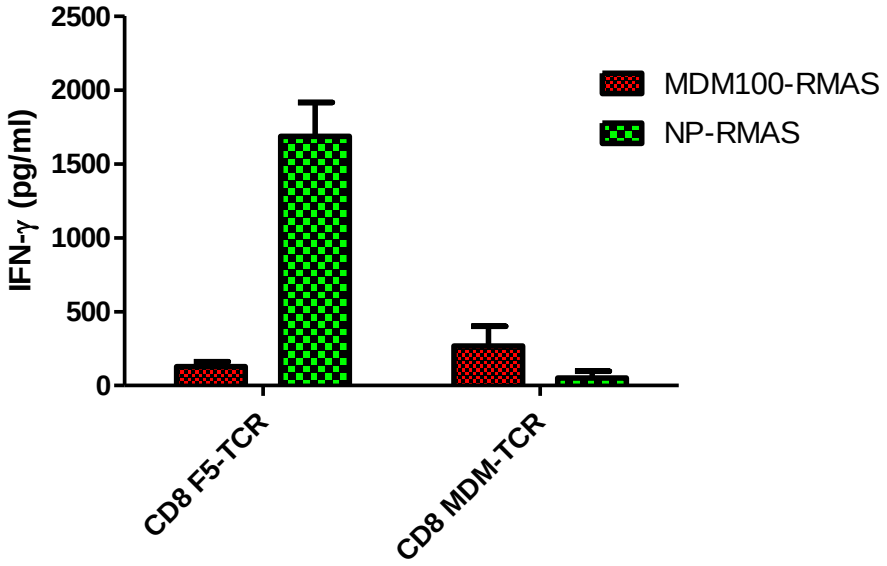


Figure 4-6 Recognition of RMAS cells loaded with saturating concentrations of peptide induces IFN-γ production by TCR transduced CD8+ T cells.
ELISA assay using wild type CD8+ T cells transduced with MDM-TCR or F5-TCR stimulated with RMA-S cells loaded with saturating concentrations (10μM) of MDM100 peptide or NP peptide in triplicate wells. This is a representative result of at least two independent experiments.

4.3 In-vitro functional analysis of CD8β-/- CD8+ T cells following co-transduction with F5-TCR and CD8β

4.3.1 Cell surface expression of CD8β-/- CD8+ T cells following transduction with F5-TCR and CD8β

Due to the limitations observed with the MDM-TCR, the index TCR used for subsequent testing of CD8 co-receptor mutants was the F5-TCR. CD8β-/- CD8α+ T cells were transduced with viral supernatant containing a 1:1 mixture of F5-TCR and the CD8β constructs either of wild-type sequence (WT) or one of the four MHC-binding site mutants (S53L, S54V, L58R and L58R/I25A) or one of the four O-glycosylation mutants (T120A, T121A, T124A and T120A/T121A/T124A [TglyM]). The controls were CD8β-/- CD8α+ T cells mock transduced (unmodified) or transduced with the F5-TCR alone. Three days after transduction, the transduced T cells were analysed for surface expression of F5-TCR and CD8β using fluorochrome conjugated antibodies to the Vβ11 and to CD8β (Fig 4-7 to 4-9). As transduction was with two separate vectors, there were two distinct populations seen, a single-transduced Vβ11+ population and a double-transduced CD8β+ Vβ11+ population. The single-transduced Vβ11+ CD8α+ T cells comprised between 12.81% and 19.96% of total CD8+ T cells in all conditions except mock negative control. This indicates slight variation in transduction efficiency as the same volume of a common F5-TCR supernatant mix was used in the different conditions. The double-transduced CD8β+ Vβ11+ population comprised between 13.40% and 20.74% of total CD8+ T cells in all conditions except mock negative control and the F5-TCR alone condition. Similar to transduction shown above using the F5-TCR, most CD8β+ transduced T cells were also Vβ11+. The expression level of CD8β as measured by MFI was similar between WT (MFI: 280) and the different mutations (MFI: 276 to 368) with once again S53L having the lowest MDI expression (238). These expression features were consistent between at least three repeat transductions.

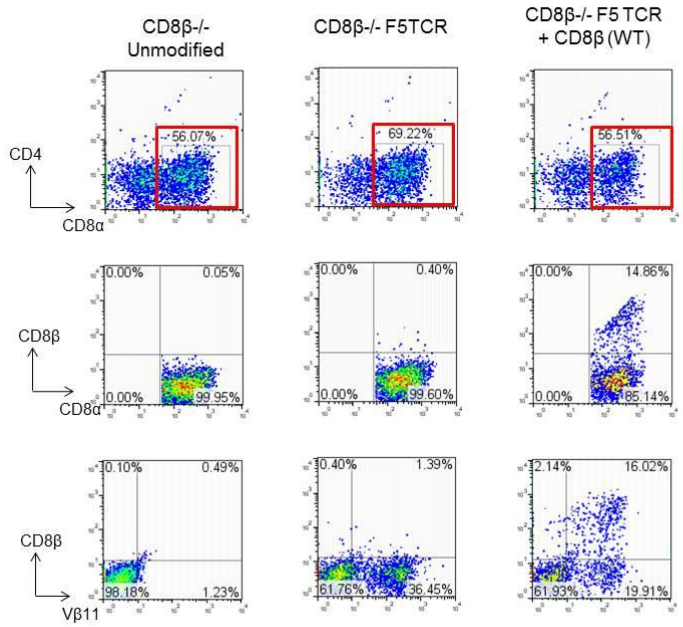


Figure 4-7 FACS analysis following F5-TCR and CD8β (WT) transduction of CD8β-/- CD8+ T cells.
Mock transduced CD8β-/- CD8+ T cells (unmodified) were used as control for gating purposes. The first row show that the sorted live lymphocytes were predominantly CD8α+ T cells which were gated (red square) and examined for expression of CD8β and TCR (Vβ11) on subsequent rows. Each column represents a separate condition.

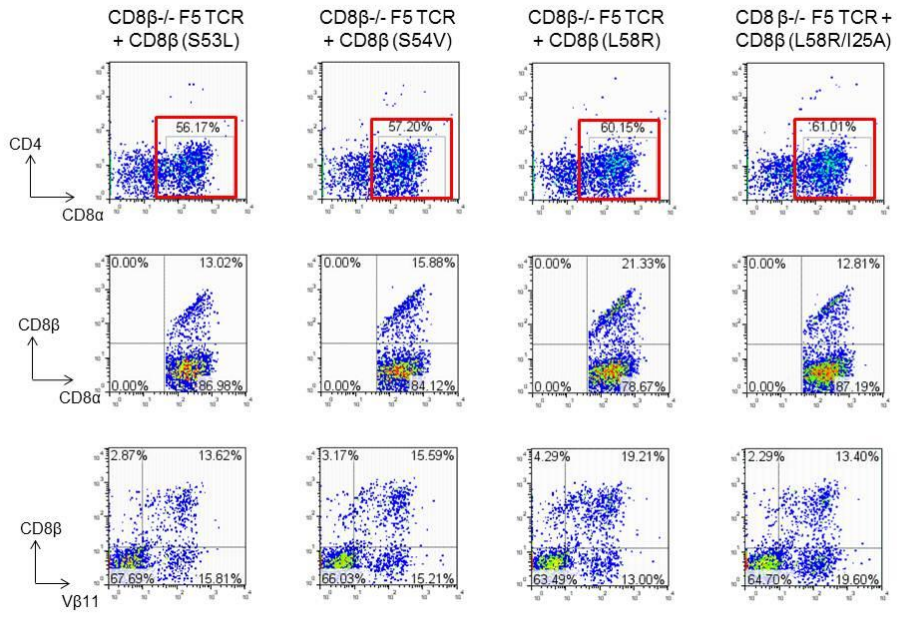


Figure 4-8 FACS analysis following transduction of CD8β-/- CD8+ T cells with the F5-TCR vector and CD8β vector containing MHC-binding site mutations (S53L, S54V, L58R or L58R/I25A).
Mock transduced CD8β-/- CD8+ T cells (unmodified) were used as control for gating purposes. The first row show that the sorted live lymphocytes were predominantly CD8α+ T cells which were gated (red square) and examined for expression of CD8β and TCR (Vβ11) on subsequent rows. Each column represents a separate condition.

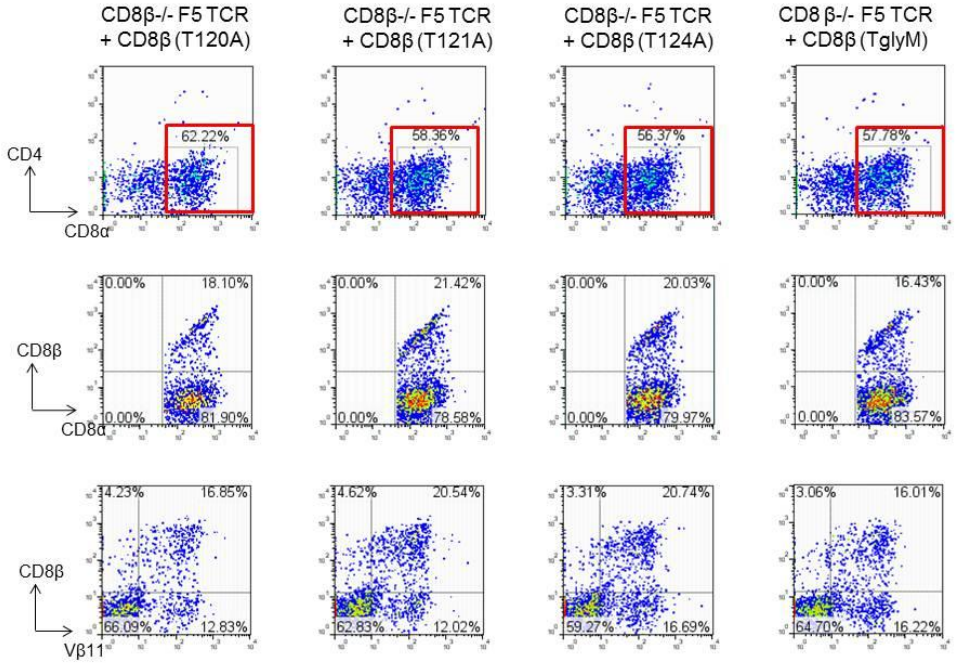


Figure 4-9 FACS analysis following transduction of CD8β-/- CD8+ T cells with the F5-TCR vector and CD8β vector containing glycosylation site mutations (T120A, T121A, T124A, and TglyM).
Mock transduced CD8β-/- CD8+ T cells (unmodified) were used as control for gating purposes. The first row show that the sorted live lymphocytes were predominantly CD8α+ T cells which were gated (red square) and examined for expression of CD8β and TCR (Vβ11) on subsequent rows. Each column represents a separate condition.

Cell sorting was performed to enrich the CD8β+ population using anti-CD8β-FITC antibodies and anti-FITC magnetic beads (Figure 2.3.3.1). The enriched T cell populations were re-stimulated and rested for one week to reduce the effects of anti-CD8β antibodies on functional comparisons of the CD8β transduced cells. The control 'F5-TCR alone' transduced T cells were re-stimulated without any enrichment.

After 1 round of stimulation, the different cell populations were analysed by FACS before use in functional assays. The FACS analyses (Figure 4-10 to 4-12) showed significant enrichment of the CD8β+ Vβ11+ doubly transduced CD8+ T cells (80.34% - 91.15% purity) except for the CD8β(S54V) transduced cells which had a purity post bead-sort and re-stimulation of 58.76%. The MFI for the transduced molecules were similar in the different cell populations; CD8β MFI varied between 176 and 222 and Vβ11 MFI varied between 119 and 123.

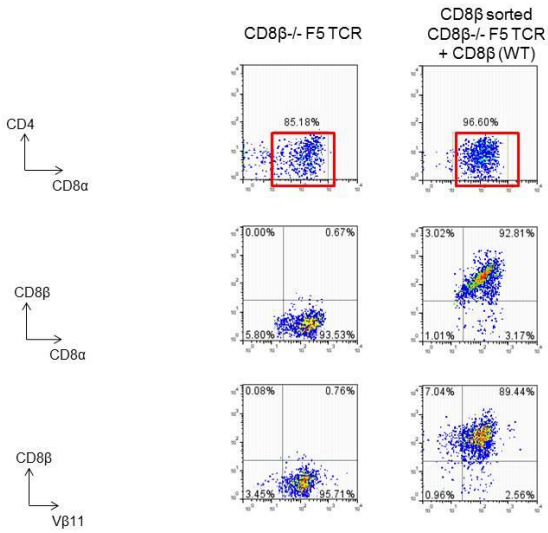


Figure 4-10 Enrichment of F5-TCR and CD8β (WT) transduced CD8β-/- CD8+ T cells. Sorted TCR (Vβ11+) transduced lymphocytes were analysed 7 days after in-vitro peptide stimulation. F5-TCR (Vβ11+) transduced CD8β-/- CD8+ T cells (unmodified) were used for gating purposes. The first row show that the sorted live lymphocytes were predominantly CD8α+ T cells which were gated (red square) and examined for expression of CD8β and TCR (Vβ11) on subsequent rows. Each column represents a separate condition.

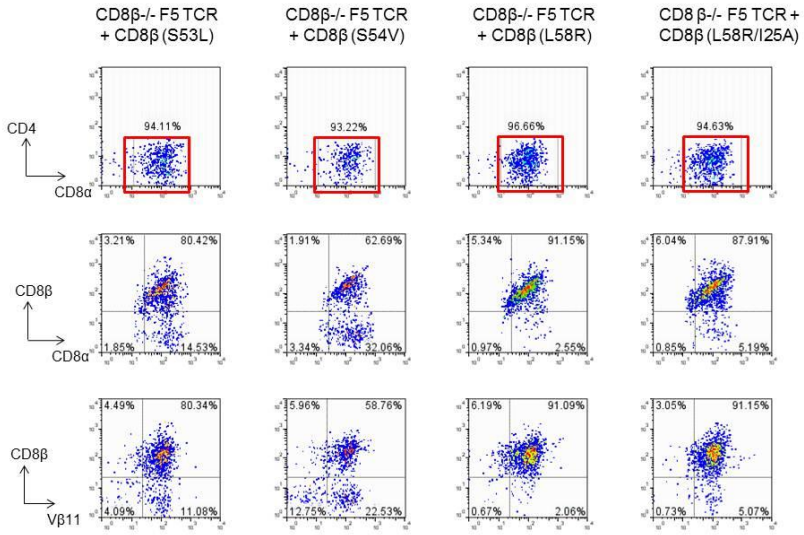


Figure 4-11 Enrichment of the F5-TCR and CD8β transduced CD8β-/- CD8+ T cells containing CD8β MHC-binding site mutations (S53L, S54V, L58R and L58R/I25A). Sorted TCR (Vβ11+) transduced lymphocytes were analysed 7 days after in-vitro peptide stimulation. F5-TCR (Vβ11+) transduced CD8β-/- CD8+ T cells (unmodified) were used for gating purposes. The first row show that the sorted live lymphocytes were predominantly CD8α+ T cells which were gated (red square) and examined for expression of CD8β and TCR (Vβ11) on subsequent rows. Each column represents a separate condition.

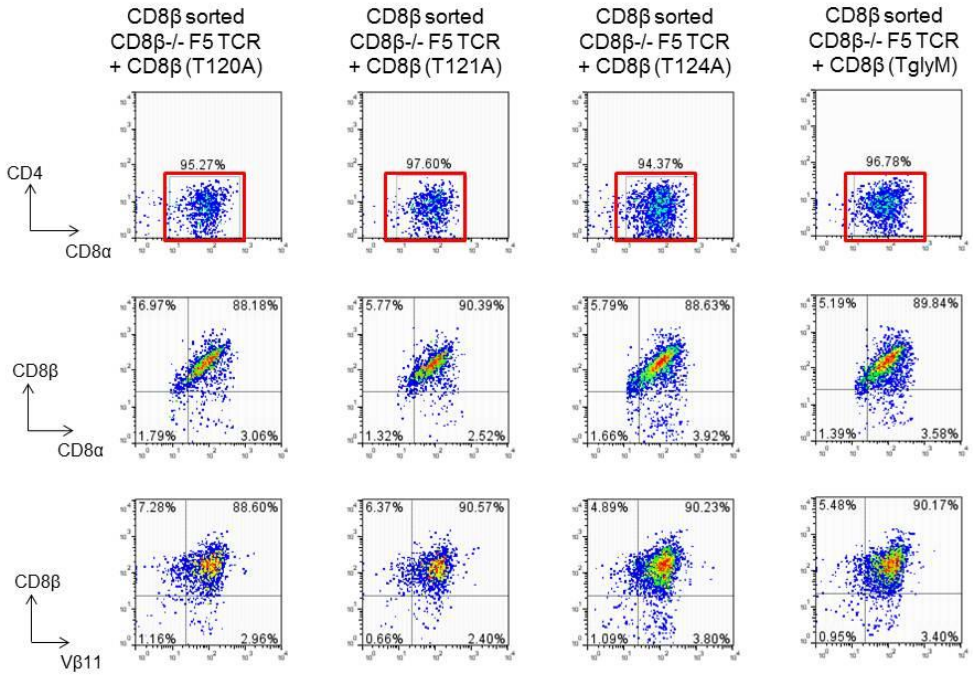


Figure 4-12 Enrichment of F5-TCR and CD8β transduced CD8β-/- CD8+ T cells containing CD8β glycosylation site mutations (T120A, T121A or T124A and TglyM). Sorted TCR (Vβ11+) transduced lymphocytes were analysed 7 days after in-vitro peptide stimulation. F5-TCR (Vβ11+) transduced CD8β-/- CD8+ T cells (unmodified) were used for gating purposes. The first row show that the sorted live lymphocytes were predominantly CD8α+ T cells which were gated (red square) and examined for expression of CD8β and TCR (Vβ11) on subsequent rows. Each column represents a separate condition.

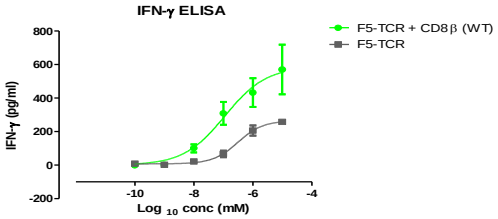
4.3.2 Antigen specific IFN-γ production of F5-TCR transduced CD8β-/- CD8+ T cells

To determine the effect of mutated CD8β co-receptor expression in F5-TCR-td CD8+ T cells, the same number of purified CD8β+ and F5-TCR double transduced CD8+ T cells were incubated with peptide loaded RMAS target cells, with a range of peptide concentration from 10μM to 100pM. After overnight stimulation, supernatant was removed and IFN-γ production measured by ELISA. These results were used to derive a log dose response curve generated using Prism5 (Graphpad). F5-TCR-td T cells expressing the CD8β MHC-binding site mutants (Fig 4-13) and CD8β glycosylation site mutants (Fig 4-14) were compared to those expressing CD8β wild-type (WT) in two different sets of experiments.

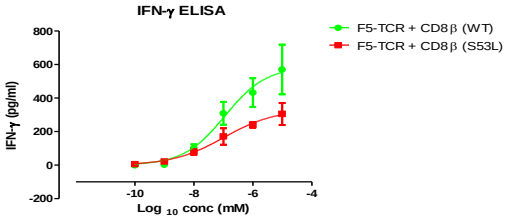
The introduction of CD8β (WT) enhanced the maximal Ag-specific IFN-γ production of F5-TCR-td CD8+ T cells (587 vs 266 pg/ml) and also increased the functional avidity, that is the T cells recognised lower peptide concentration (logEC50: -6.947 vs -6.509). Of the eight CD8β mutants tested, only the CD8β (L58R) and CD8β (L58R/I25A) mutants demonstrated enhanced in comparison to the CD8β (WT) F5-TCR-td CD8+ T cells. CD8+ cells transduced with the CD8β (L58R) mutant and the CD8β (L58R/I25A) mutant had more than twice the maximal IFN-γ response

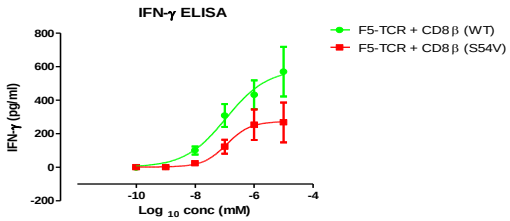
compared with CD8+ T cells transduced with CD8β (WT) (2072 and 1567 vs 586 pg/ml). Moreover, the cells transduced with the CD8β (L58R) and CD8β (L58R/I25A) mutants recognised lower concentrations of relevant NP peptide than CD8+ T cells transduced with CD8β WT (logEC50: -7.276 and -7.131 vs -6.947). Both the CD8β (S53L) and CD8β (S54V) mutants were inferior to the CD8β (WT) in augmenting the avidity of F5-TCR-td CD8+ T cells.

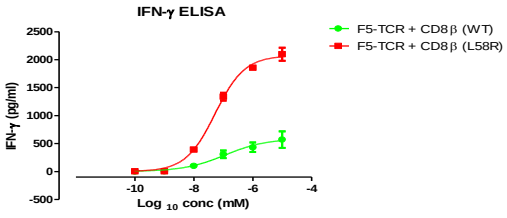
CD8β co-receptor constructs with single glycosylation site mutations (T120A, T121A, or T124A) did not alter IFN-γ production and peptide sensitivity in response to peptide loaded RMAS cells compared to equivalent T cells expressing the CD8β (WT) co-receptor (Fig 4-14). However, the CD8β mutant containing all three glycosylation site mutations (TglyM) was inferior to the CD8β (WT) when transduced into F5-TCR-td CD8+ T cells as determined by a lower maximal Ag-specific IFN-γ production (1532 vs 1910 pg/ml) and a decrease in relevant peptide sensitivity (logEC50: -7.965 vs -8.186).



CD8β-/- CD8 T cells	logEC50	EC50
F5-TCR	-6.503	3.137e-07
F5-TCR + CD8β (WT)	-6.947	1.131e-07
F5-TCR + CD8β (S53L)	-6.979	1.049e-07
F5-TCR + CD8β (S54V)	-6.927	1.183e-07
F5-TCR + CD8β (L58R)	-7.276	5.303e-08
F5-TCR + CD8β (L58R/I25A)	-7.131	7.394e-08







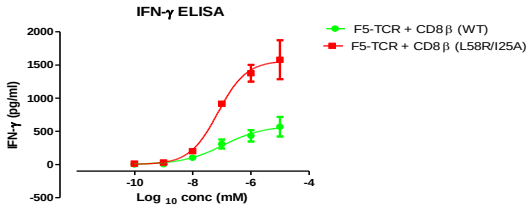
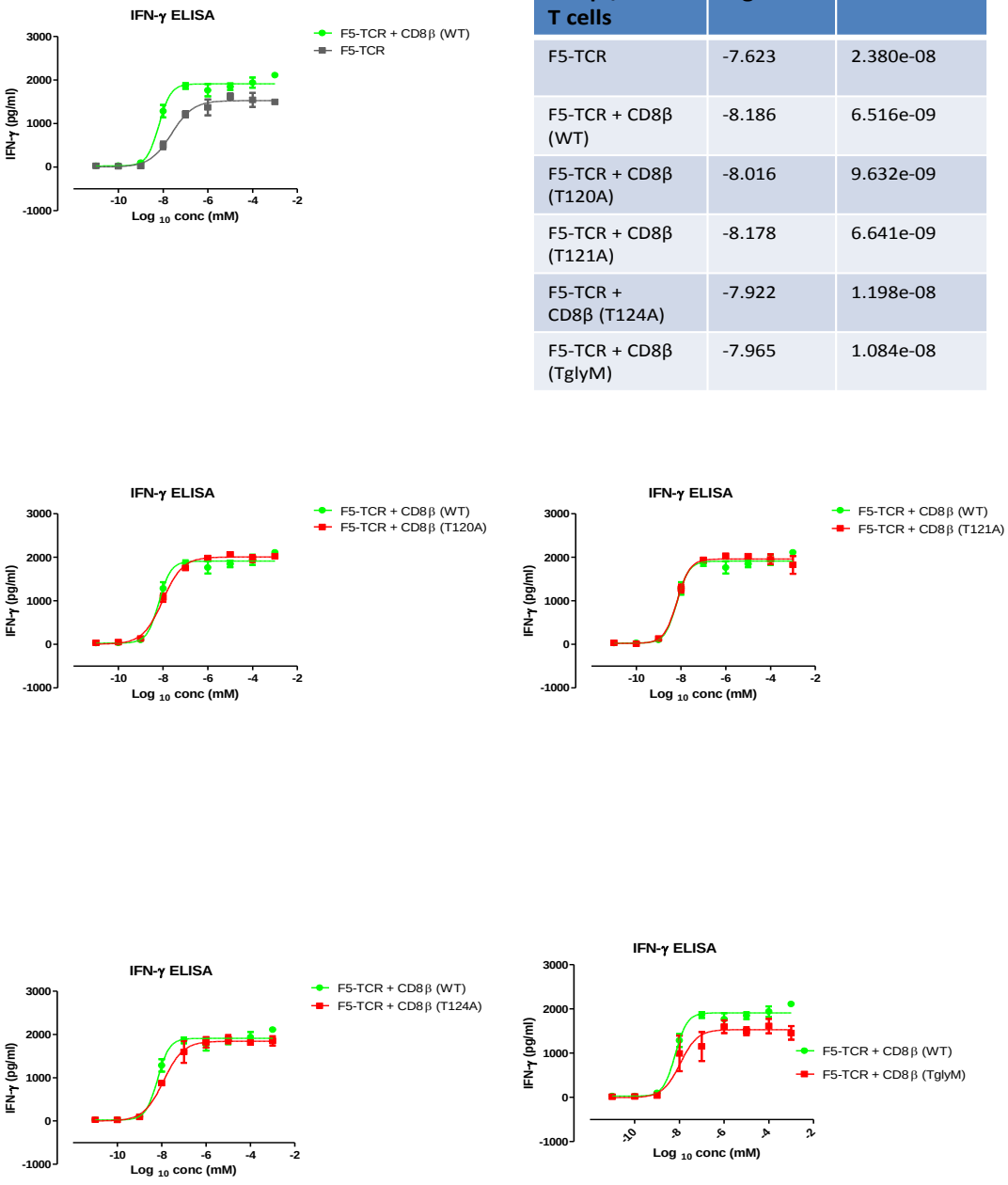


Figure 4-13 In vitro functional avidity of F5-TCR CD8β-/- CD8+ T cells is augmented by CD8β L58R and L58R/I25A mutations compared to CD8β wild-type (WT).
Ag-specific IFN-γ secretion was determined by ELISA following overnight stimulation with RMAS cells loaded with different concentrations of relevant NP peptide. The NP peptide concentration required to elicit a response halfway between maximum and minimum (EC50) is shown in the table on the top right. This is a representative result of at least two independent experiments.



CD8β-/- CD8 T cells	logEC50	EC50
F5-TCR	-7.623	2.380e-08
F5-TCR + CD8β (WT)	-8.186	6.516e-09
F5-TCR + CD8β (T120A)	-8.016	9.632e-09
F5-TCR + CD8β (T121A)	-8.178	6.641e-09
F5-TCR + CD8β (T124A)	-7.922	1.198e-08
F5-TCR + CD8β (TglyM)	-7.965	1.084e-08

Figure 4-14 In vitro functional avidity of F5-TCR CD8β-/- CD8+ T cells is not augmented by CD8β glycosylation site mutations (T120A, T121A, T124A or TglyM) compared to CD8β wild-type (WT).

Ag-specific IFN-γ secretion was determined by ELISA following overnight stimulation with RMAS cells loaded with different concentrations of relevant NP peptide. The NP peptide concentration required to elicit a response halfway between maximum and minimum (EC50) is shown in the table on the top right. This is a representative result of at least two independent experiments.

Further experiments were performed where the various F5-TCR transduced T cell populations were stimulated with tumour cells endogenously expressing the NP peptide. The transduced T cells were incubated overnight with EL4NP target or EL4 control tumour cells. All F5-TCR-td CD8+ T cells only recognized EL4NP target and not EL4 control tumour cells, confirming appropriate recognition of the NP epitope through the F5-TCR. The CD8β L58R and CD8β L58R/I25A mutated co-receptors were used in these experiments as they had previously been shown to enhance IFN-γ responses to peptide-loaded RMAS cells. All F5-TCR-td CD8+ T cells produced five to ten fold more IFN-γ in response to EL4NP target cells than EL4 control tumour cells (Fig 4-15). The introduction of CD8β (WT) enhanced the Ag-specific IFN-γ production of F5-TCR-td CD8+ T cells (589 vs 266 pg/ml; p=0.0043). CD8+ T cells transduced with the CD8β (L58R) mutant and the CD8β (L58R/I25A) mutant produced significantly more IFN-γ compared with CD8+ T cells transduced with CD8β (WT) (1672 and 1315 vs 589 pg/ml; p=0.022 and p=0.026) in response to NP-expressing tumour cells.

Therefore the CD8β L58R mutated co-receptor, when expressed alongside the F5-TCR was consistently superior to the CD8β wild-type co-receptor in augmenting the Ag-specific IFN-γ production by F5-TCR-td CD8+ T cells. As the CD8β (L58R) mutant enhanced effector function marginally better than the CD8β (L58R/I25A) mutation it was chosen as the key 'augmenting mutant' for subsequent experiments described in Chapters 5 and 6.

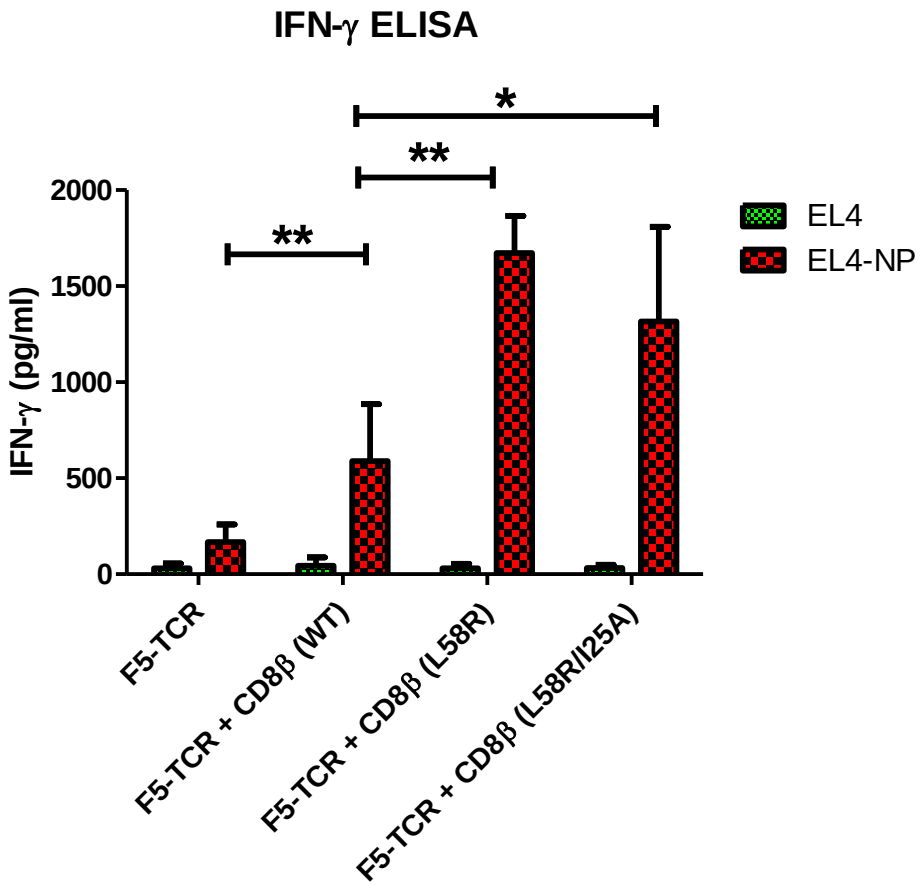


Figure 4-15 Higher Ag-specific IFN-γ production by CD8β L58R and L58R/I25A compared to CD8β wild-type (WT) co-transduced F5-TCR CD8α+ T cells to EL4NP tumour cells which express endogenously processed NP peptide.
IFN-γ ELISA assay using CD8β-/- CD8+ T cells transduced with F5-TCR vector and CD8β vector containing wild-type (WT) or MHC-binding site mutant (L58R or L58R/I25A) were incubated with EL4NP target or EL4 control tumour cells for 16 hours with each condition replicated six times. This is a representative result of at least two independent experiments. (* $p < 0.05$, ** $p < 0.01$).

4.4 Summary

This chapter has described the in vitro analysis of T cells transduced with the CD8β mutants discussed in Chapter 3. The effect of CD8β mutant expression was tested in CD8β-/- CD8+ T cells. The early experiments used the 'CD8-dependent' MDM-TCR which recognises the MDM100 peptide sequence YAMIYRNL from the tumour oncogene Murine double-minute 2 (MDM) in the context of H-2Kb. While the MDM-TCR and CD8β expression in co-transduced T cells were reasonable (Fig 4-1 to 4-3), the function of transduced T cells were sub-optimal; the overall IFN-γ production was low and MDM peptide-specific responses were poor (Fig 4-4 and 4-5). Therefore comparison of the effect of CD8β variants on peptide specific responses was not possible with the MDM-TCR.

As the cloned MDM-TCR was unsuitable it was replaced with the F5-TCR for the remainder of this project. The F5-TCR recognizes the NP366 peptide sequence ASNENMDAM from the Influenza-A nucleoprotein (NP) and is also known to be dependent on the CD8 co-receptor for optimal function (Morris et al. 2005; Jiang et al. 2011). CD8β-/- CD8+ T cells were co-transduced with the F5-TCR and the CD8β variants (Fig 4-7 to 4-9). These transduced cells were sorted using antibodies to CD8β and analysed one week post antigen-specific stimulation (Fig 4-10 to Fig 4-12). The F5-TCR-td T cells expressing the CD8β L58R mutation, CD8β (L58R), were able to augment T-cell response above that observed with T cells expressing the CD8β with wild-type sequence, CD8β (WT) (Fig 4-13 and Fig 4-14). This enhancement of Ag-specific IFN-γ production was also seen in response to EL4 tumour cells endogenously expressing the NP peptide (Fig 4-15).

Chapter 5

Chapter 5. In-vitro analysis in CD4+ and CD8+ T cells

5.1 Introduction

The previous chapter demonstrated that introducing CD8β molecules with wild-type sequence or containing the L58R mutation improved the function F5-TCR transduced into transgenic CD8β-/- CD8+ T cells. In this chapter CD8β co-transfer into wild-type C57Bl/6 T cells is examined. In CD8+ T cells there is physiological expression of endogenous CD8 co-receptor and it is unknown if the introduction of additional CD8β molecules containing augmenting mutations will confer additional benefits.

In CD4+ T cells, the addition of CD8α molecules is required for the expression of the CD8β chain and therefore the complete CD8 transgene needs to be transferred. As summarised in Table 1-1, the MHCI restricted F5-TCR and CD8 molecule had been previously transduced together into CD4+ T cells and examined in a model of viral infection (Kessels et al. 2006). The co-transfer of CD8α with the F5-TCR into CD4+ T cells had mixed results on the recognition of tumour cells (Morris et al. 2005); improving Ag-specific IFN-γ production but abolishing proliferative responses.

To study the effects of CD8 molecules and MHCI restricted TCR on CD4+ T cells, peptide loaded splenocytes expressing both MHCI and MHCII and EL4 tumour cells, which express MHCI only were used as in-vitro targets. Of the panel of mutated CD8β constructs generated only the CD8β L58R 'augmenting' mutant and the 'less effective' CD8β TglyM mutant were studied in detail.

In order to investigate the possibility that the homologous human I59R mutation could also be augmenting, in vitro experiments were designed using human CD4 T cells transduced with MHCI restricted CMV-TCR. This TCR has been recently shown by other members of the laboratory to be CD8 dependent in terms of cytokine production and cytotoxicity. Human CD4+ T cells co-transduced with CMV-TCR and CD8 coreceptor could protect NOD/SCID mice against tumour (Xue et al. 2013).

5.2 In-vitro characterisation of F5-TCR and CD8 co-receptor transduced CD8+ T cells

5.2.1 Transduction of CD8+ T cells with the F5-TCR-CD8β vector

As wild-type C57Bl/6 CD8+ T cells express endogenous CD8β, it was not possible to directly monitor the introduction of CD8β mutants. The F5-TCR-CD8β (WT) vector was already available (Section 3.5.3) and CD8+ T cells co-transduced with the F5-TCR and CD8β molecules could be identified by staining for Vβ11 expression assuming both transgenes were expressed simultaneously. To confirm, CD8β-/- CD8+ T cells were used to validate co-expression of both F5-TCR and CD8β. Transduction with the F5-TCR-CD8β (WT), F5-TCR-CD8β (L58R) or F5-TCR-CD8β

(TglyM) vectors resulted in proportional expression of Vβ11+ and CD8β with Vβ11^{hi} transduced cells also CD8^{hi} (Fig 5-1).

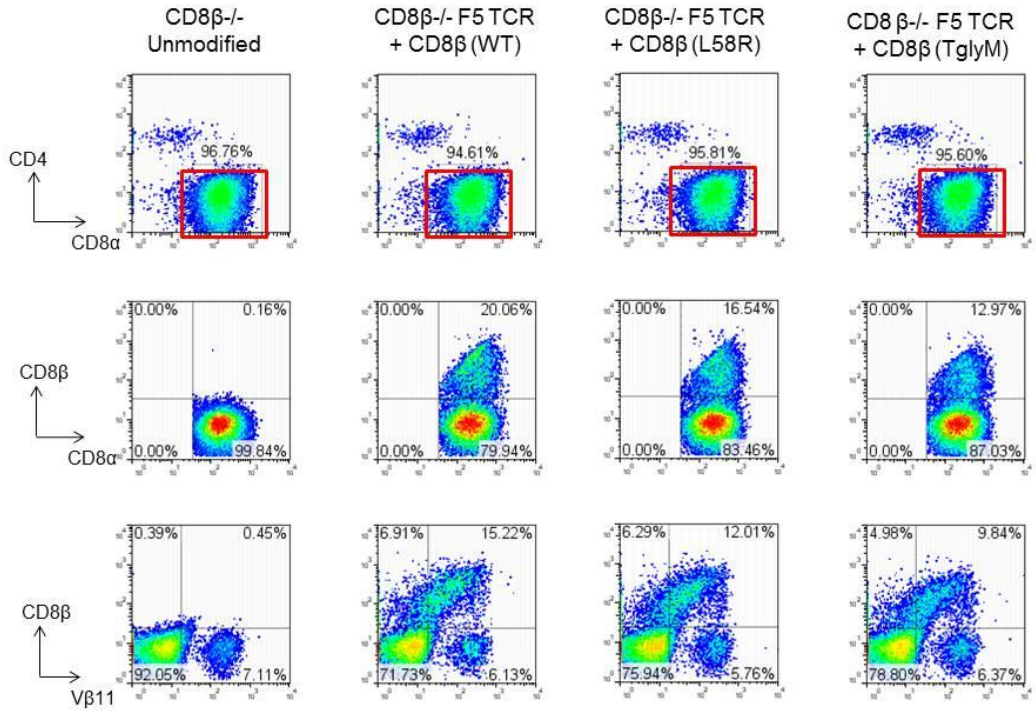


Figure 5-1 FACS analysis of transduced CD8β-/- CD8+ T cells using F5-TCR-CD8β vector containing CD8β (WT), CD8β L58R or CD8β TglyM.
Mock transduced CD8 T cells (unmodified) were used as controls for gating purposes. The first row show that the sorted live lymphocytes were predominantly CD4+ T cells which were gated (red square) and examined for expression of CD8 and TCR (Vβ11) on subsequent rows. Each column represents a separate condition.

5.2.2 Cell surface expression of F5-TCR and CD8β on transduced CD8+ T cells

Wild-type C57Bl/6 splenocytes enriched for CD8+ T cells (Section 2.3.2.1) were transduced with F5-TCR and CD8β (WT, L58R or TglyM) using the F5-TCR-CD8β vectors. FACS analysis three days post transduction (Fig 5-2) showed a similar level of F5-TCR transduction, as determined by Vβ11 expression (42.01%, 36.54% and 38.64%). Endogenous Vβ11 expression was 9.71% in the mock transduced CD8+ control T cells.

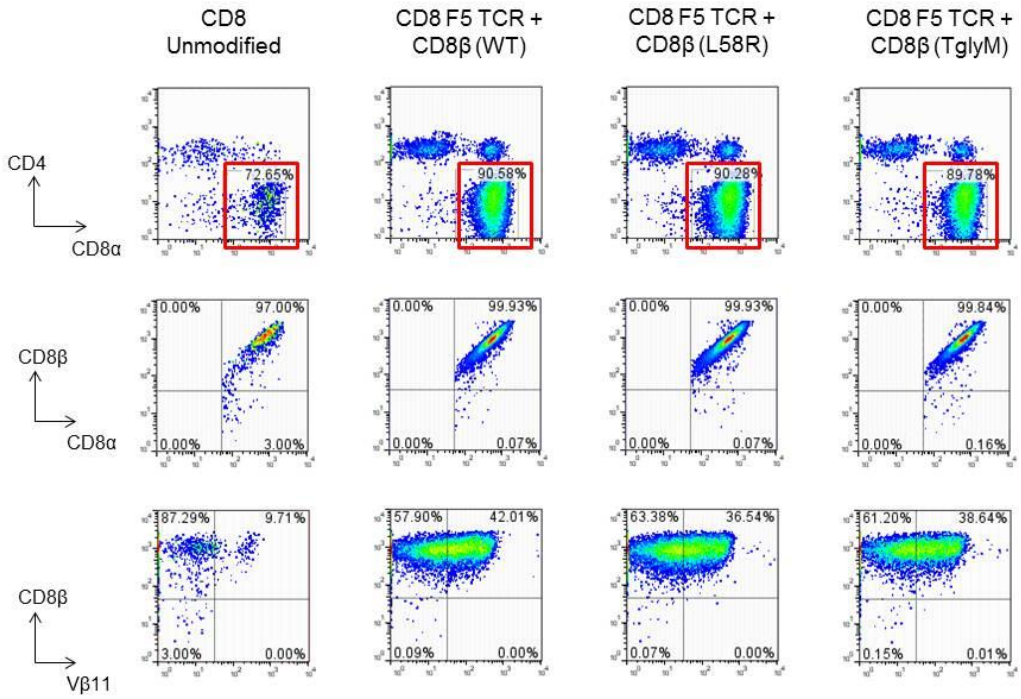


Figure 5-2 FACS analysis of transduced CD8+ T cells using F5-TCR-CD8β vector containing CD8β (WT or L58R or TglyM).
Mock transduced CD8+ T cells (unmodified) were used as controls for gating purposes. The first row show that the sorted live lymphocytes were predominantly CD8+ T cells which were gated (red square) and examined for expression of CD8β and TCR (Vβ11) on subsequent rows. Each column represents a separate condition.

5.2.3 In vitro Ag-specific cytokine production of transduced CD8+ T cells

To compare the functional avidity of F5-TCR-td CD8+ T cells, expressing additional CD8β wild-type or mutants, F5-TCR-CD8β (WT), F5-TCR-CD8β (L58R) or F5-TCR-CD8β (TglyM) transduced CD8+ T cells were incubated with splenocytes loaded with a range of NP peptide concentration from 10μM to 1nM. After overnight incubation, supernatant was analysed for IFN-γ and IL-2 production by ELISA (Fig 5-3 and 5-4). The CD8β (L58R) and (TglyM) transduced T cells were compared with CD8β (WT). No significant differences in IFN-γ and IL-2 production were observed.

To test for in vitro recognition of endogenously expressed cognate antigen the F5-TCR-CD8β (WT), F5-TCR-CD8β (L58R) or F5-TCR-CD8β (TglyM) transduced CD8+ T cells were incubated with EL4NP or EL4 control tumour cells. After overnight incubation, supernatant was removed and analysed for the amount of IFN-γ and IL-2 produced by ELISA (Fig 5-5 and 5-6). No significant difference in cytokine production was observed between the CD8β (WT) and CD8β (L58R) transduced T cells, however

the CD8β (TglyM) IFN-γ and IL-2 response to EL4NP tumour cells was significantly impaired (p=0.03).

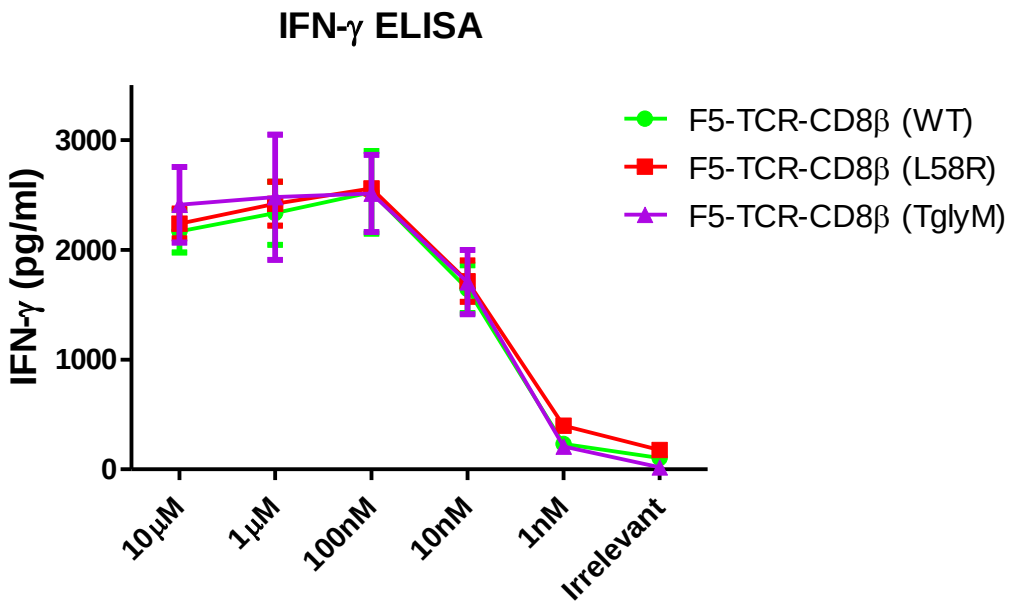


Figure 5-3 In vitro Ag-specific IFN-γ production of transduced CD8+ T cells showed no difference between CD8β wild-type or mutants (L58R and TglyM).
CD8+ T cells transduced with F5-TCR-CD8β vector containing wild-type (WT), L58R or TglyM mutations were incubated with splenocytes loaded with different concentrations of relevant NP peptide. A representative result of at least two independent experiments is shown.

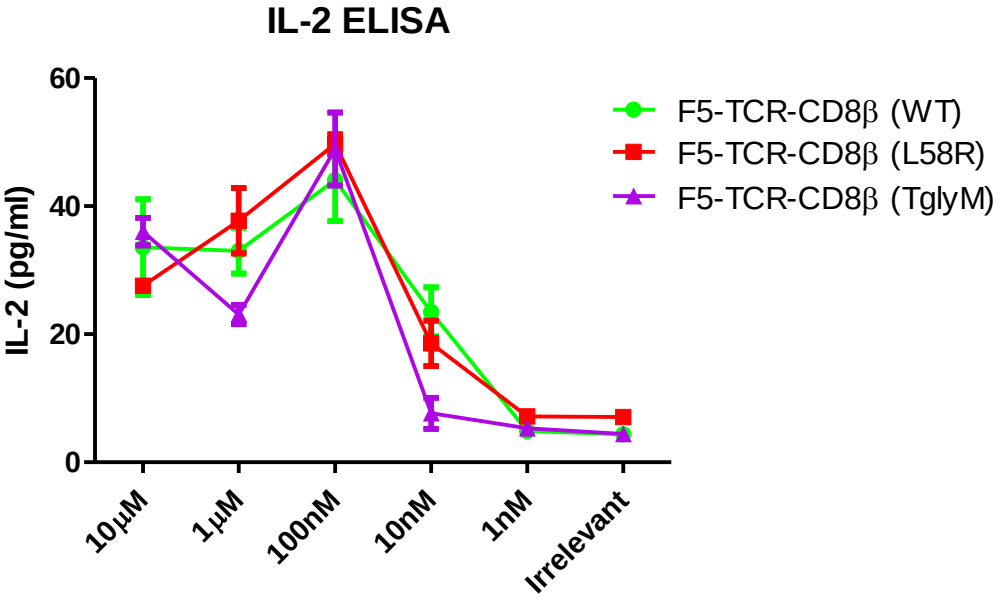


Figure 5-4 In vitro Ag-specific IL2 production of transduced CD8+ T cells showed no difference between CD8β wild-type or mutants (L58R and TglyM).
CD8+ T cells, transduced with F5-TCR-CD8β vector containing wild-type (WT), L58R or TglyM mutations were incubated with splenocytes loaded with different concentrations of relevant NP peptide. A representative result of at least two independent experiments is shown.

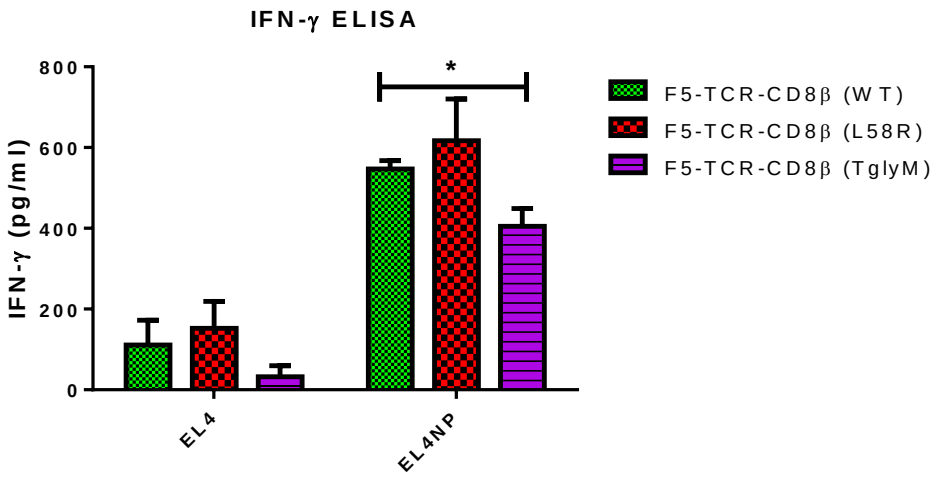


Figure 5-5 Recognition of EL4NP tumour cells expressing endogenously processed NP peptide induces IFN-γ production by F5-TCR transduced CD8+ T cells that was inferior in CD8β TglyM compared to CD8β wild-type or L58R.
CD8+ T cells, transduced with F5-TCR-CD8β vector containing wild-type (WT), L58R or TglyM mutations were incubated with EL4NP target or EL4 control tumour cells. IFN-γ production was measured by ELISA. A representative result of at least two independent experiments is shown. (*p<0.05).

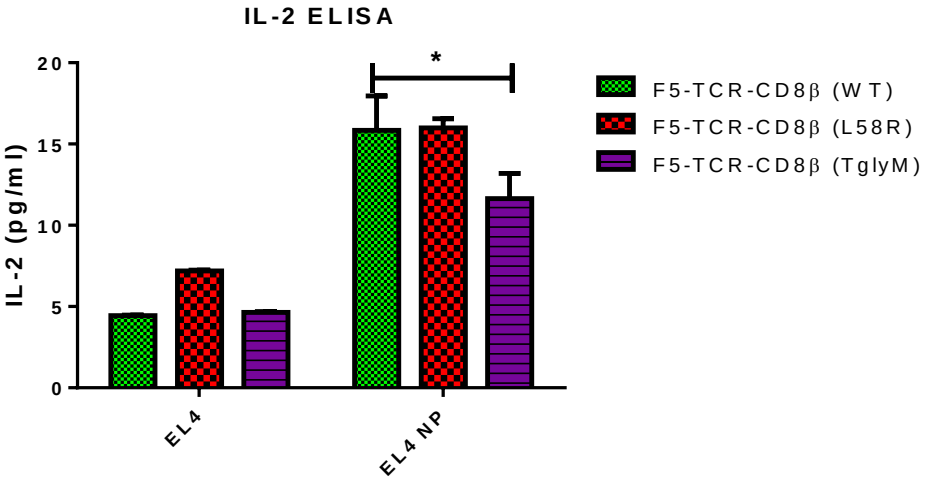


Figure 5-6 Recognition of EL4NP tumour cells expressing endogenously processed NP peptide induces IL-2 production by F5-TCR transduced CD8+ T cells that was inferior in CD8β TglyM compared to CD8β wild-type or L58R.
CD8+ T cells, transduced with F5-TCR-CD8β vector containing wild-type (WT), L58R or TglyM mutations were incubated with splenocytes loaded with EL4NP target or EL4 control tumour cells. IL-2 production was measured by ELISA. A representative result of at least two independent experiments is shown. (*p<0.05).

Intracellular cytokine staining (ICS) was also performed to determine the proportion of F5-TCR-CD8β (WT), F5-TCR-CD8β (L58R) or F5-TCR-CD8β (TglyM) transduced CD8+ T cells that produced Ag-specific responses to EL4NP or EL4 tumour cells (Fig 5-7). The experimental set up was similar to the ELISA assay detailed above, except that the cells were incubated for a shorter 6 hours prior to permeabilisation and

fixation (Section 2.5.4). ICS showed that there were no significant differences observed between CD8β (WT) and CD8β (L58R) and CD8β (TglyM) transduced T cell secretion of IFN-γ (13.82%, 13.90% vs 10.64%) or IL-2 (3.33%, 3.03% vs 2.78%).

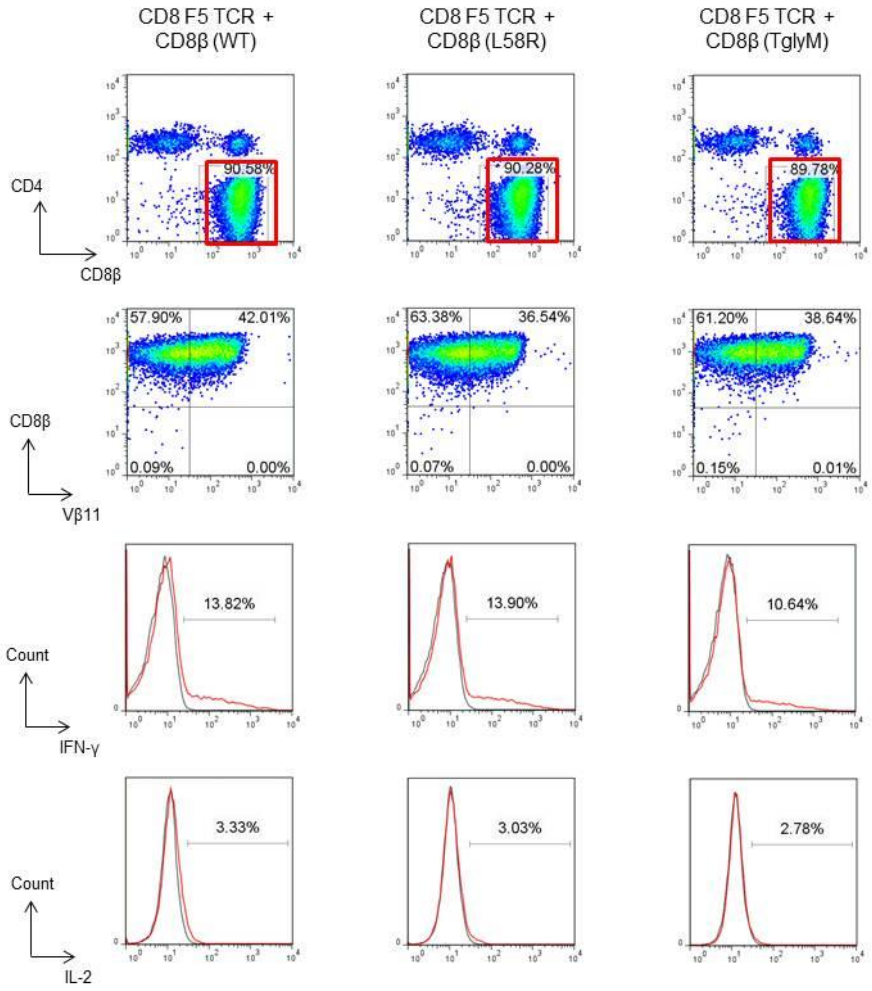


Figure 5-7 In vitro Ag-specific intracellular IFN-γ and IL-2 production to EL4NP tumour cells by transduced CD8+ T cells was inferior in CD8β TglyM compared to CD8β WT or L58R .
Intracellular cytokine staining was performed after incubation of transduced CD8+T cells with EL4NP (red line) target or EL4 control (grey line) tumour cells for 6 hours. Transduced CD8+ T cells were gated (red squares) and analysed for IFN-γ and IL-2 production in respective columns. This result is representative of at least two independent experiments.

5.3 In-vitro characterisation of F5-TCR and CD8 co-receptor transduced CD4+ T cells

5.3.1 Cell surface expression of F5-TCR and CD8 co-receptor on transduced CD4+ T cells

Wild-type C57Bl/6 splenocytes enriched for CD4+ T cells (Section 2.3.2.2) were transduced with F5-TCR and CD8αβ molecules containing CD8β wild-type (WT) or CD8β mutations (L58R or TglyM) using separate vectors. FACS analysis three days after transduction (Fig 5-8) showed a similar level of F5-TCR transduction by Vβ11 expression (73.64%, 69.43% and 67.57% respectively) compared with mock transduced or F5-TCR alone transduced CD8+ T cells expressing endogenous Vβ11 (7.27% and 86.74% respectively). For the three conditions with transduction of CD8 (WT), CD8 (L58R) and CD8 (TglyM), the percentage of CD8α+ CD8β+ T cells were similar at 50.01%, 49.17% and 48.02% respectively. The cell surface expression of CD8α molecules was proportional to the CD8β molecules indicating heterodimer formation. The CD8α expression (as measured by MFI) was similar between the three conditions (MFI: 746, 795, 893) as was the CD8β expression (MFI: 301, 265, 257).

$CD8^{hi}$ expressing T cells were enriched using anti-CD8α-APC antibodies and anti-APC microbeads to ensure similar numbers of T cells expressing the transduced CD8 co-receptor were compared in functional assays. The anti-CD8α-APC antibody was derived from the KT15 clone and is known not to augment or diminish Ag-specific T cell responses in subsequent functional assays (Devine et al. 2004).

FACS analysis (Fig 5-9) sorted CD4+ T cells showed significant enrichment of the double transduced CD8+ Vβ11+ CD4+ T cells with 84.22% (CD8WT), 82.06% (CD8L58R), and 78.65% (CD8TglyM) of total cells expressing both the co-receptor and the F5-TCR. The observed MFI for F5-TCR expression (as measured by Vβ11+ staining) were similar in the three T cell populations (MFI: 581, 529 and 538). The sorted cells were also stained pentamer (ASNENDAM/H-2Db). The co-transfer of CD8 co-receptor with the F5-TCR was shown to enhance pentamer binding compared to introduction of F5-TCR alone (MFI: 316, 315, 307 vs 233).

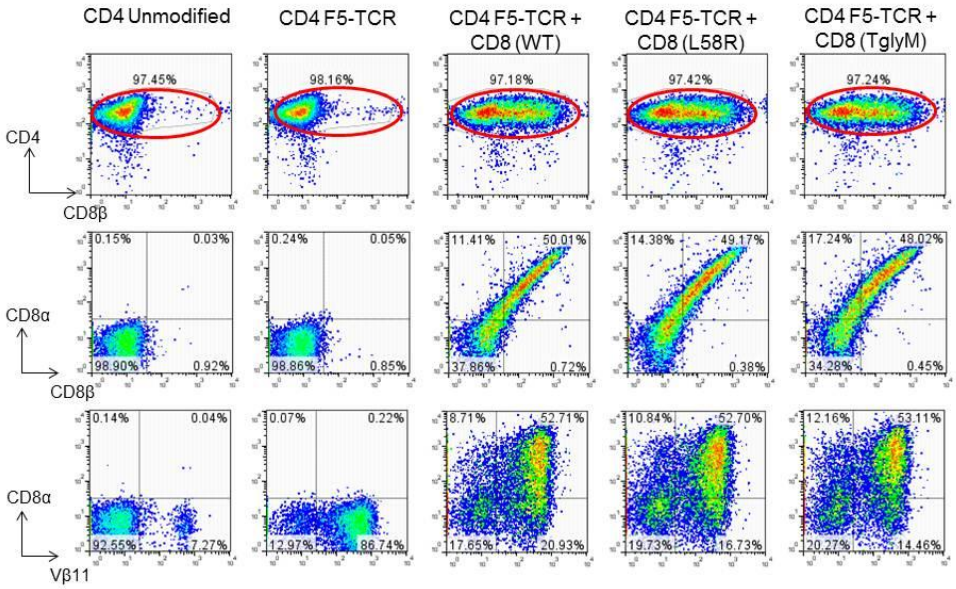


Figure 5-8 F5-TCR and CD8 co-receptor expression of transduced CD4+ T cells.
Mock transduced CD4 T cells (unmodified) were used as controls for gating purposes. The first row show that the sorted live lymphocytes were predominantly CD4+ T cells which were gated (red circle) and examined for expression of CD8 and TCR (Vβ11) on subsequent rows. Each column represents a separate condition.

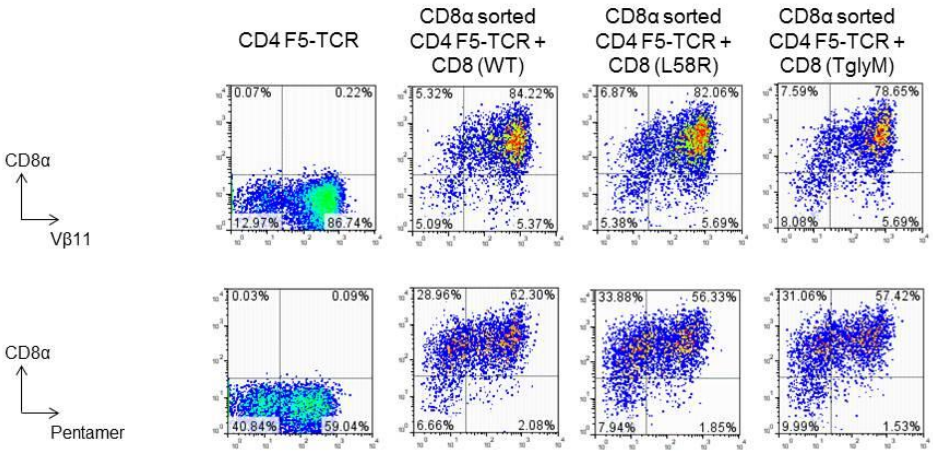


Figure 5-9 F5-TCR and CD8 co-receptor expression of transduced CD4+ T cell populations was similar between CD8 wild-type and mutants.
The sorted live lymphocytes from Fig 5-8 were examined for expression of CD8, TCR (Vβ11) and H2Db/NP pentamer staining on subsequent rows. Each column represents a separate condition.

5.3.2 In vitro Ag-specific cytokine secretion of transduced CD4+ T cells

Functional avidity of the different transduced T cell populations was compared using peptide titration experiments, where the target cells were peptide loaded syngeneic splenocytes. The double transduced CD4+ T cells enriched for CD8α were stimulated with congenic splenocytes loaded with a range of NP peptide concentration from 1 μM to 100pM. 'F5-TCR alone' transduced CD4+ T cells were included as control. After overnight incubation, supernatant was analysed for IL-2 and IFN-γ by ELISA (Fig 5-10 and 5-11). F5-TCR-td CD4+ T cells without CD8 molecules produced little IL-2 or IFN-γ to splenocytes pulsed with the different NP peptides concentrations. CD4+ T cells transduced with CD8 (L58R) produced more IL-2 than those transduced with CD8 (WT), especially at lower NP peptide concentrations, but there was little difference in IFN-γ secretion. The CD4+ T cells transduced with CD8 (TglyM) produced less IL-2 and IFN-γ than those with CD8 (WT).

Recognition of tumour cells endogenously expressing cognate antigen was tested by stimulation of the transduced CD4+ T cells with EL4NP target or EL4 control tumour cells. 'F5-TCR alone' transduced CD4+ T cells were included as a control. After overnight incubation, supernatant was removed and analysed for IL-2 and IFN-γ by ELISA (Fig 5-12 and 5-13). CD4+ T cells transduced with CD8 (L58R) had significantly higher Ag-specific IL-2 production than those transduced with CD8 (WT) (62.67 vs 38.84 pg/ml; p=0.0022). No significant difference in IFN-γ secretion was observed. The CD4+ T cells transduced with CD8 (TglyM) produced less IL-2 (32.8 vs 38.8 pg/ml) and IFN-γ (62.0 vs 159 pg/ml) than those expressing CD8 (WT).

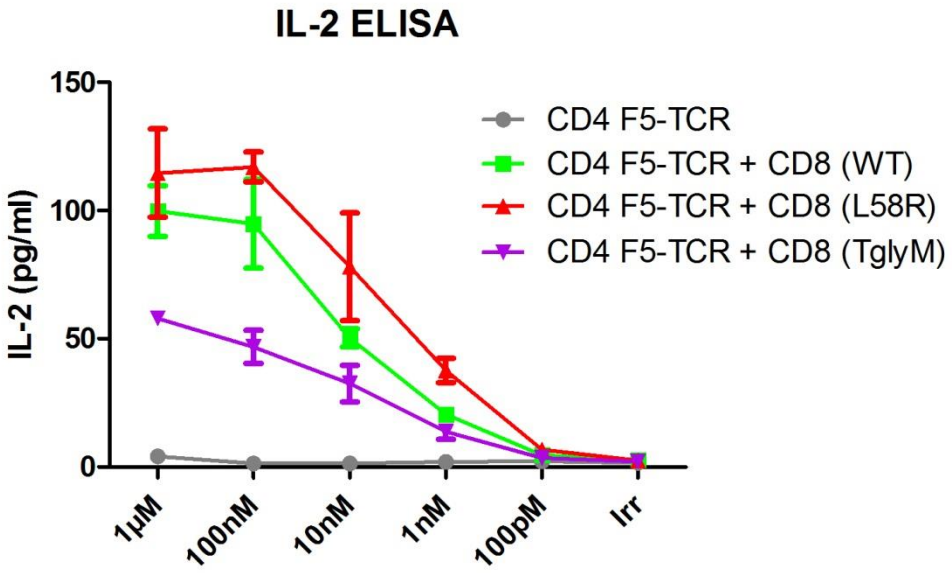


Figure 5-10 In vitro IFN-γ functional avidity of F5-TCR and CD8 transduced CD4+ T cells was higher in CD8 L58R compared to CD8 WT and TglyM.
CD4+ T cells, transduced with F5-TCR and CD8 vectors containing CD8β wild-type (WT), L58R or TglyM mutations were incubated with splenocytes loaded with different concentrations of relevant NP peptide or irrelevant SV9 peptide and supernatant examined for IL-2 secretion was measured by ELISA. This result is representative of at least two independent experiments.

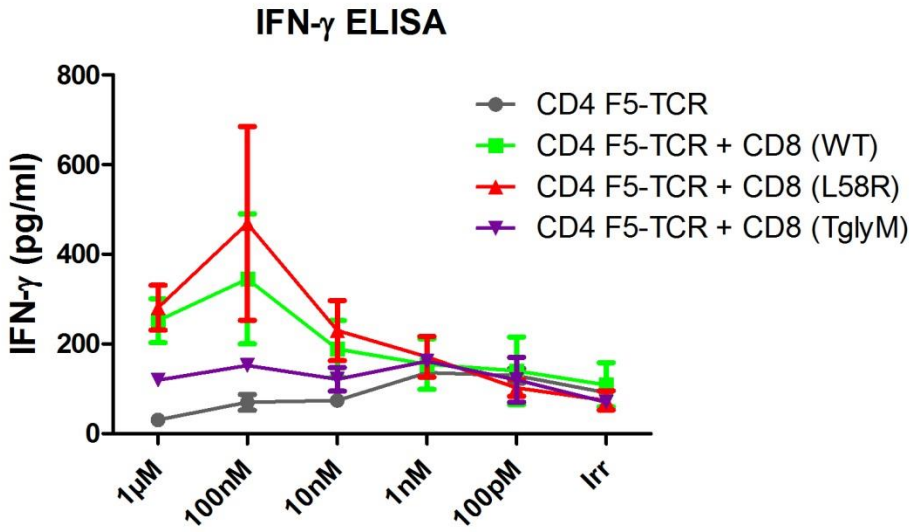


Figure 5-11 In vitro IL-2 functional avidity of F5-TCR and CD8 co-receptor T cells was similar between CD8 wildtype and mutants.
CD4+ T cells, transduced with F5-TCR and CD8 vectors containing CD8β wild-type (WT), L58R or TglyM mutations were incubated with splenocytes loaded with different concentrations of relevant NP peptide or irrelevant SV9 peptide and supernatant examined for IFN-γ secretion was measured by ELISA. This result is representative of at least two independent experiments.

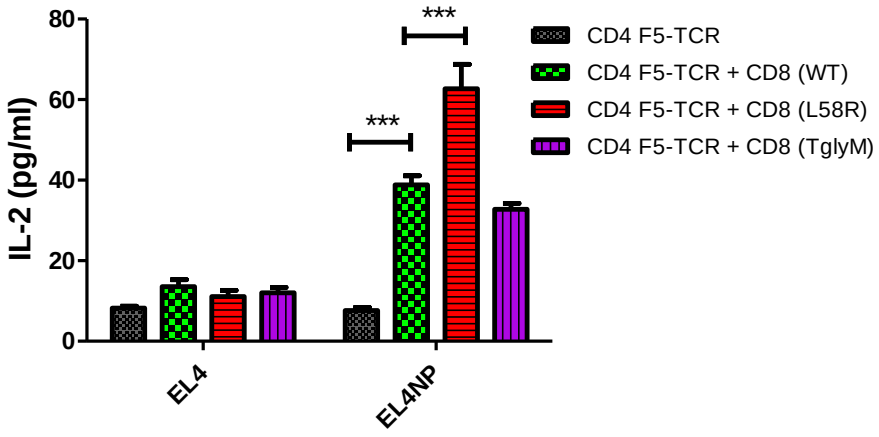


Figure 5-12 Ag-specific IL-2 production by F5-TCR transduced CD4+ T cells was highest in CD8 L58R compared to CD8 wild-type and TglyM against EL4NP tumour cells expressing endogenously processed NP peptide.
CD4+ T cells transduced with F5-TCR and CD8 vectors containing CD8β wild-type (WT), L58R or TglyM mutations were incubated with EL4NP target or EL4 control tumour cells for 16 hours and supernatant examined for IL-2 secretion was measured by ELISA. This result is representative of at least two independent experiments. (*** p <0.005).

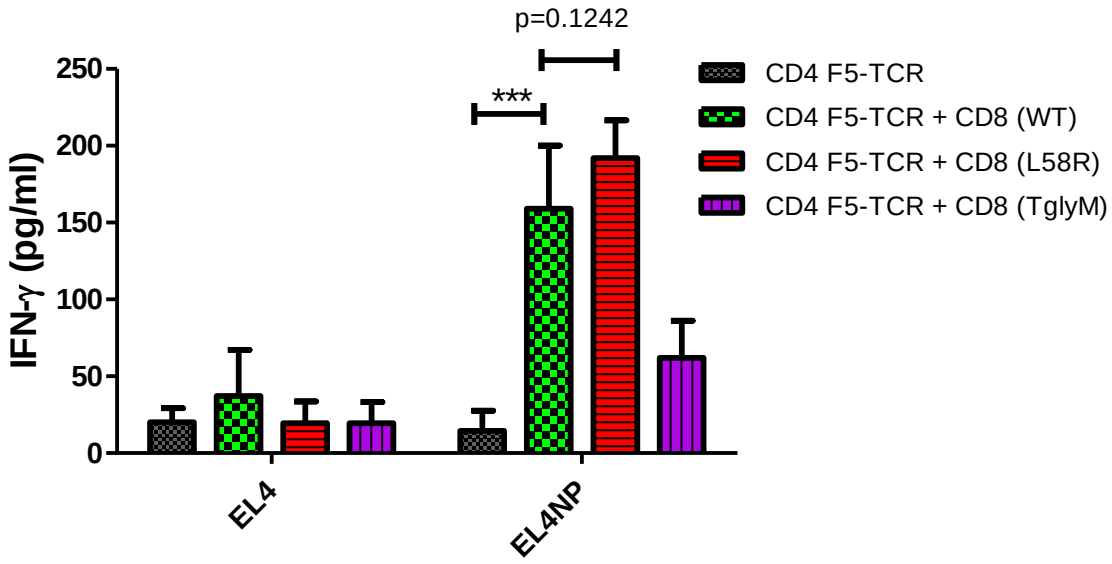


Figure 5-13 Ag-specific IL-2 production by F5-TCR transduced CD4+ T cells was highest in CD8 L58R compared to CD8 wild-type and TglyM against EL4NP tumour cells expressing endogenously processed NP peptide.
CD4+ T cells transduced with F5-TCR and CD8 vectors containing CD8β wild-type (WT), L58R or TglyM mutations were incubated with EL4NP target or EL4 control tumour cells for 16 hours and supernatant examined for IFN-γ secretion was measured by ELISA. This result is representative of at least two independent experiments. (*** p <0.005).

Intracellular cytokine staining (ICS) was also performed to determine the proportion of F5-TCR-td CD4+ T cells co-transduced with CD8 (WT), CD8 (L58R) or CD8 (TglyM) that produce Ag-specific responses to EL4NP or EL4 tumour cells. The set up was similar to the ELISA assay except that the cells were incubated for a shorter 6 hours before permeabilisation and fixation (Section 2.5.4). ICS demonstrated that only the CD8+ Vβ11+ double-transduced CD4+ T cells displayed Ag-specific production of IL-2 and IFN-γ and up-regulation of CD40L. Transduced CD4+ T cells expressing CD8 (L58R) compared with CD8 (WT) had greater Ag-specific production of IL-2 (27.79% vs 18.69%) and IFN-γ (3.51% vs 2.97%) and enhanced up-regulation of CD40L (63.42% vs 53.17%). Although 'F5-TCR alone' transduced CD4+ T cells produced poor Ag-specific IFN-γ (0.49%) and IL-2 (0.49%), the up-regulation of CD40L was more obvious (41.74%).

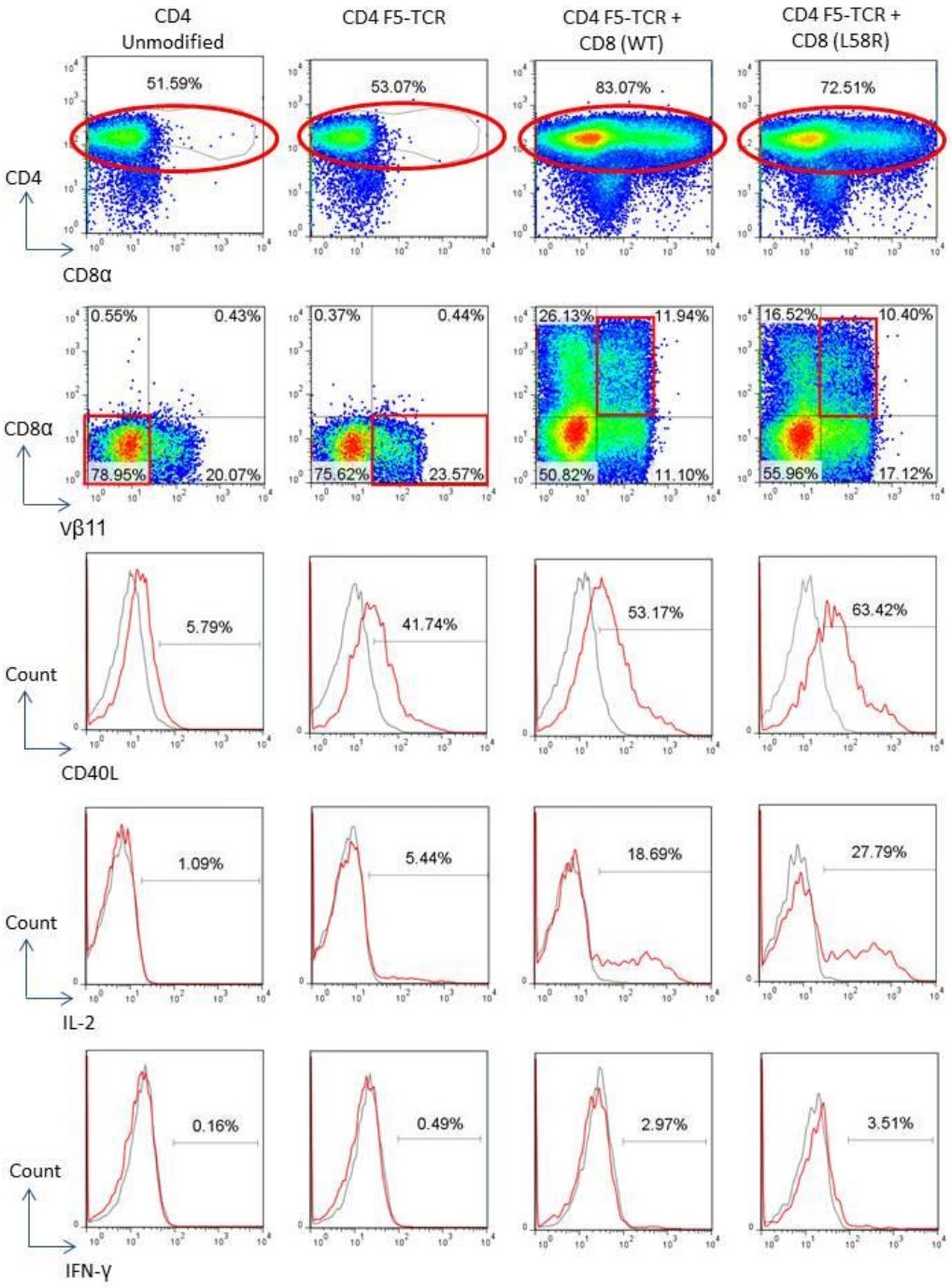


Figure 5-14 In vitro Ag-specific intracellular IFN-γ and IL-2 production by F5-TCR transduced CD4+ T cells production was highest in CD8 L58R compared to CD8 wild-type and TglyM against EL4NP tumour cells expressing endogenously processed NP peptide.

Intracellular cytokine staining was performed after incubation of transduced CD4+T cells with EL4NP (red line) target or EL4 control (grey line) tumour cells for 6 hours. Conditions of untransduced (Vβ11-/CD8α-) (first column red square) or F5-TCR alone (Vβ11+) (second column red square) or double transduced (Vβ11+/CD8α+) (third/fourth column red square) CD4+ T cells were gated and analysed for IFN-γ and IL-2 production in subsequent rows. This result is representative of at least two independent experiments.

5.3.3 In-vitro cytotoxicity of F5-TCR and CD8 co-receptor transduced CD4+ T cells

Transgenic and transduced CD4+ T cells had been shown by others to target tumour cells through direct cytotoxicity (Section 1.5.2). In vitro cytotoxicity experiments were performed to compare the ability of the F5-TCR and CD8 co-receptor transduced CD4+ T cells to kill tumour cells. CD4+ T cells enriched for CD8α were incubated with chromium labelled EL4NP target tumour cells. 'F5-TCR alone' transduced CD4+ T cells were included as a control. The cells were incubated for four hours and supernatant removed to determine the amount of chromium released. Without CD8 molecules, F5-TCR-td CD4+ T cells induced tumour lysis only at the highest 50:1 effector/target (E:T) ratio. The expression of CD8 co-receptor improved cytotoxicity, but this was markedly reduced to that observed with with F5-TCR CD8+ T cells, which were known to induce tumour lysis of >50% at E:T ratio of 50:1 (McNicol et al. 2007). No differences in cytotoxicity were observed with the different transduced CD4+ T cell populations.

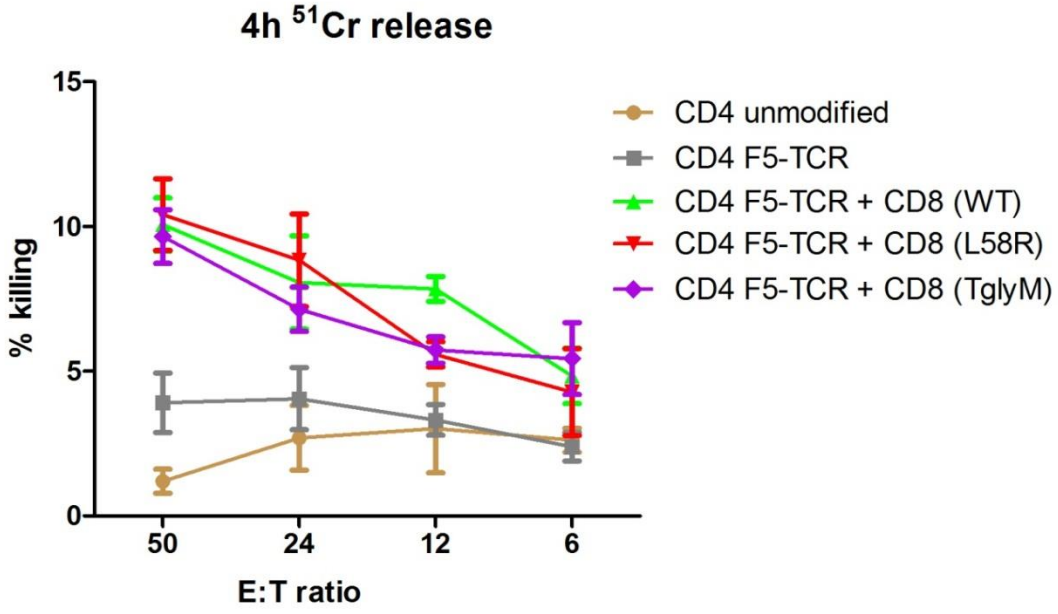


Figure 5-15 In vitro cytotoxicity of F5-TCR and CD8 transduced CD4+ T cells was similar between CD8 WT and L58R or TglyM mutants.
Sorted transduced CD4+ T cells shown in Fig 5-9 (transduction efficiency >78%) were incubated with ^{51}Cr loaded EL4NP tumour cells for 16 hours and supernatant examined for using a gamma counter. Each condition was replicated six times.

5.4 In-vitro characterisation of human CD4+ T cells following transduction of CMV-TCR and CD8 co-receptor

5.4.1 Cell surface expression of human CD4+ T cells following transduction with CMV-TCR and CD8 co-receptor

HLA-A2+ human peripheral blood mononuclear cells (PBMC) were enriched for CD4+ T cells (Section 2.3.2.3) then transduced with CMV-TCR and CD8αβ molecules containing CD8β wild-type (WT) or CD8β mutations (I59R). The human CD8β I59R mutation is homologous to the murine CD8β L58R mutation. Transduction was performed using the CMV-TCR-CD8 pMP71 vector which is a quad-cistronic vector previously produced by Dr S Xue and Dr S Ghorasian (Section 3.2). PCR mutagenesis was performed to introduce the CD8β I59R mutation as described previously for the generation of murine CD8β mutations but using human CD8β specific primers. FACS analysis three days after transduction (Fig 5-16) showed that the transduced CD4+ T cells expressed CMV-TCR as detected by antibodies to murine Cβ (constant region of the beta chain), CD8α and CD8β. The expression of CD8α molecules was proportional to the CD8β molecules indicating heterodimer formation. The CD8α expression by MFI was higher in the CD8β (WT) transduced than the CD8 (I59R) transduced T cells (MFI: 1479 vs 1057) but the CD8β expression was similar between the two conditions (MFI: 223 vs 215).

Re-stimulation with irradiated pp65 peptide loaded T2 cells and syngeneic PBMC feeder cells enhanced the enrichment of CMV-TCR-CD8 transduced CD4+ T cells. After one round of re-stimulation approximately 40% of the cells were CMV-TCR positive by staining for murine-Cβ (Fig 5-17) and following two rounds of re-stimulation around 80% of the cells were CMV-TCR positive by staining for murine-Cβ (Fig 5-18). At this stage, the expression level of the CMV-TCR was similar in the two transduced populations (MFI: 64 vs 72). The CD8α expression remained higher in the CD8 (WT) transduced T cells than the CD8 (I59R) transduced T cells (MFI: 1145 vs 733).

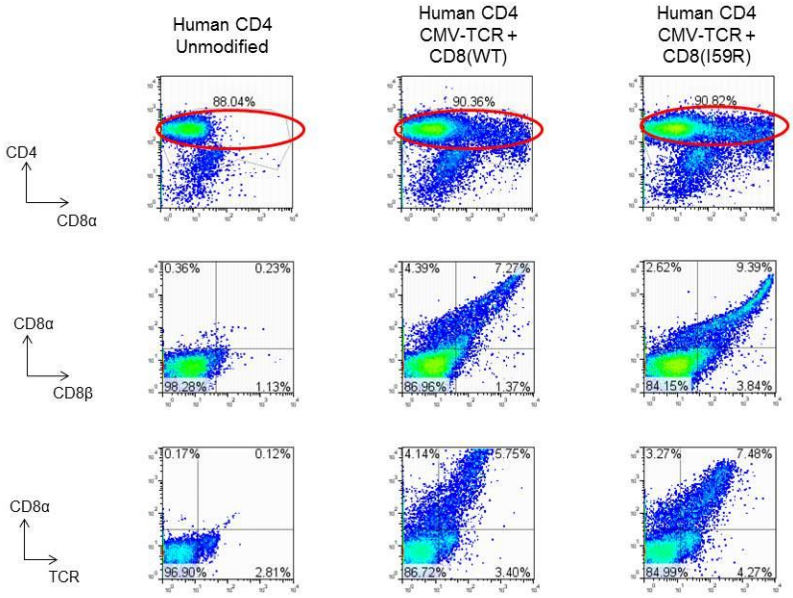


Figure 5-16 TCR and CD8 expression of human CD4+ T cells following transduction with the CMV-TCR-CD8 vector containing CD8β wild-type or I59R mutation.
Mock-transduced CD4+ T cells (unmodified) were used as controls for gating purposes. The first row show that the sorted live lymphocytes were predominantly CD4+ T cells which were gated (red circle) and examined for expression of CD8 of TCR (using murine TCR constant-β chain staining) expression on subsequent rows. Each column represents a separate condition.

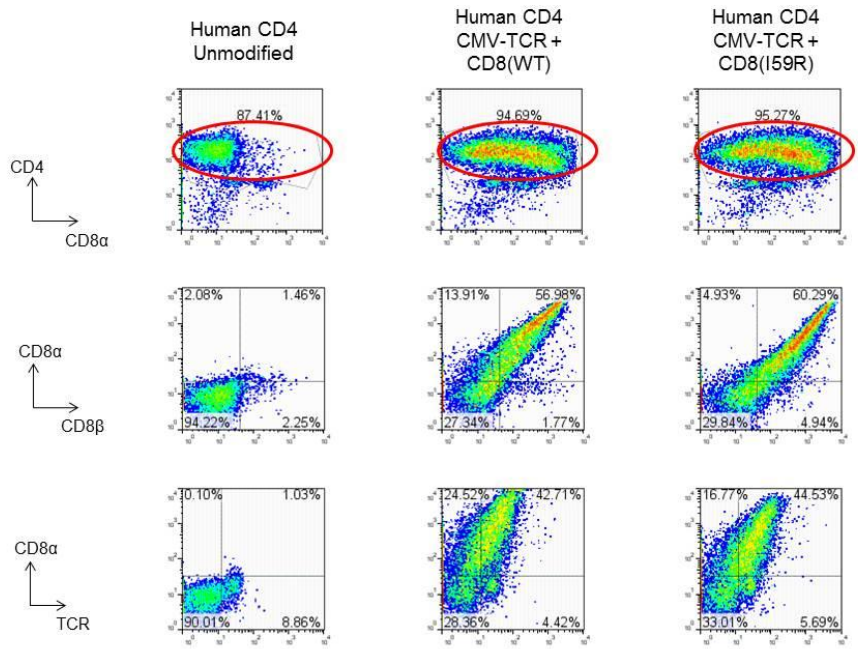


Figure 5-17 FACS analysis of CMV-TCR and CD8 transduced human CD4+ T cells after one re-stimulation show enrichment of TCR expressing cells.
Mock-transduced CD4+ T cells (unmodified) were used as controls for gating purposes. The first row show that the sorted live lymphocytes were predominantly CD4+ T cells which were gated (red circle) and examined for expression of CD8 and TCR (using murine TCR constant-β chain staining) on subsequent rows. Each column represents a separate condition.

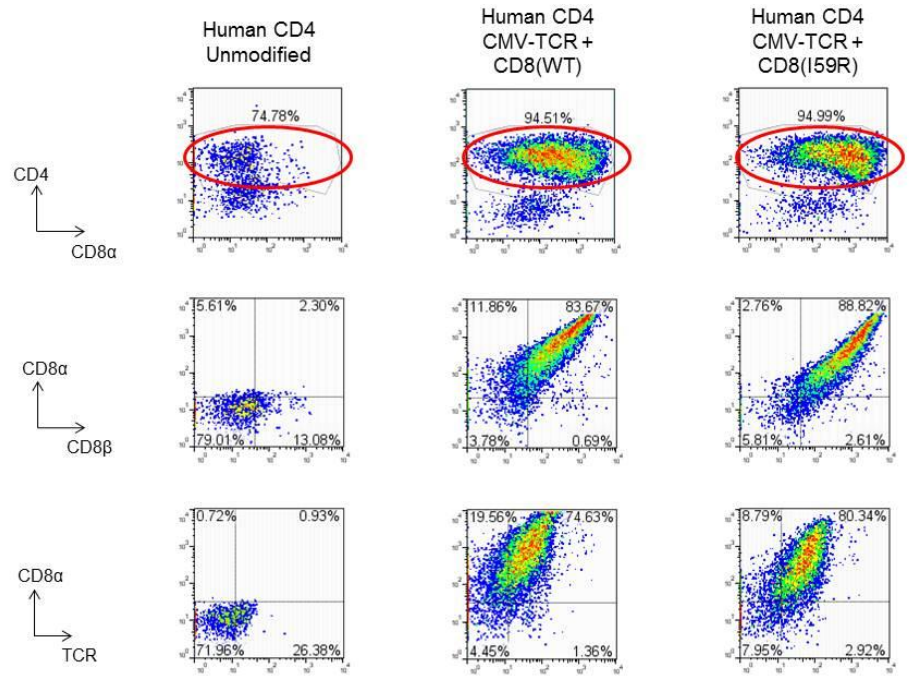


Figure 5-18 FACS analysis of CMV-TCR and CD8 transduced human CD4+ T cells after two re-stimulations show further enrichment of TCR expressing cells.
Mock-transduced CD4+ T cells (unmodified) were used as controls for gating purposes. The first row show that the sorted live lymphocytes were predominantly CD4+ T cells which were gated (red circle) and examined for expression of CD8 and TCR (using murine TCR constant-β chain staining) on subsequent rows. Each column represents a separate condition.

5.4.2 In-vitro Ag-specific cytokine secretion of transduced human CD4+ T cells

Functional avidity of the different transduced human CD4+ T cell populations were compared using peptide titration experiments, where the target cells were peptide loaded T2 cells. The re-stimulated CMV-TCR-td CD4+ T cells with CD8 (WT) or CD8 (I59R) were incubated with peptide loaded T2 cells in triplicates. The T2 cells were loaded with a range of CMVpp65 peptide concentrations from 100 nM to 10 nM. After overnight incubation, supernatant was analysed for IL-2 and IFN-γ by ELISA (Fig 5-19 and 5-20). CD4+ T cells transduced with CD8 (I59R) produced higher concentrations of IL-2 between 100 pM and 1 nM concentration but at higher peptide loading concentrations IL-2 production was similar to T cells transduced with the CD8 (WT). No difference in IFN-γ production was observed.

Recognition of tumour cells endogenously expressing cognate antigen was tested by incubating the restimulated CD4+ T cells with KA2 tumour cells transfected with pp65 (KA2pp65) or KA2 without pp65 (KA2). After overnight incubation, supernatant was analysed for IL-2 and IFN-γ by ELISA (Fig 5-21 and 5-22). No significant differences were observed between the CD8 (WT) or CD8 (I59R) transduced CD4+ T

cells with respect to Ag-specific IL-2 (655 vs 715 pg/ml; p=0.700) or IFN-γ responses (1850 vs 1673 pg/ml; p=0.658).

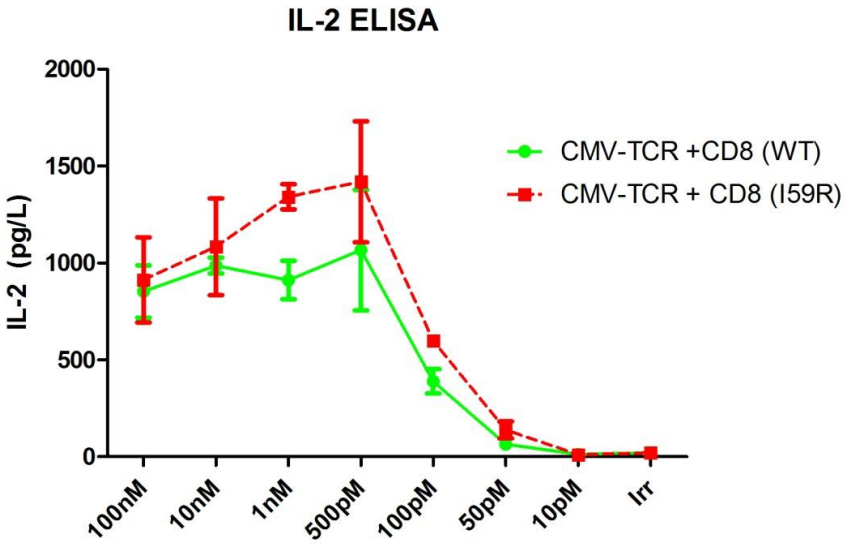


Figure 5-19 In vitro functional avidity of CMV-TCR and CD8 transduced human CD4+ T cells was similar between CD8 wild-type and I59R mutant.
CD4+ T cells, transduced with CMV-TCR-CD8 vector containing CD8β wild-type (WT) or I59R mutation were incubated with T2 cells loaded with different concentrations of relevant pp65 peptide or irrelevant (SV9) peptide. IL-2 secretion was measured by ELISA. This is a representative result of at least two independent experiments from two different human donors.

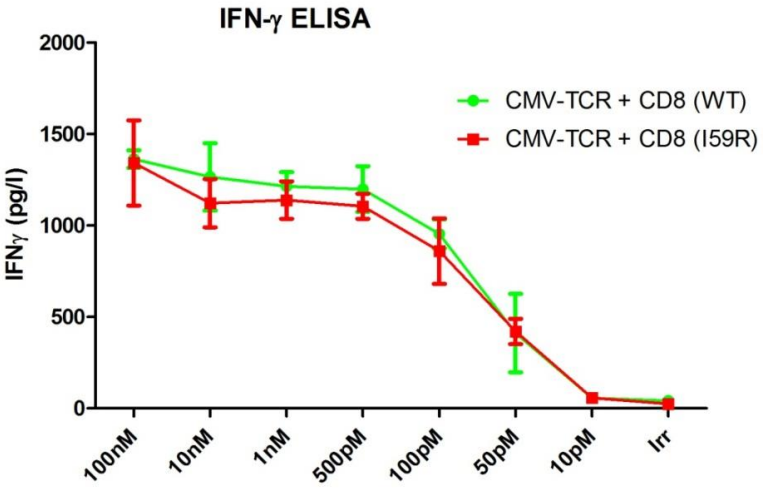


Figure 5-20 In vitro functional avidity of CMV-TCR and CD8 transduced human CD4+ T cells was similar between CD8 wild-type and I59R mutant.
CD4+ T cells, transduced with CMV-TCR-CD8 vector containing CD8β wild-type (WT) or I59R mutation were incubated with T2 cells loaded with different concentrations of relevant pp65 peptide or irrelevant (SV9) peptide. IFN-γ secretion was measured by ELISA. Shown is a representative result of at least two independent experiments from two different human donors.

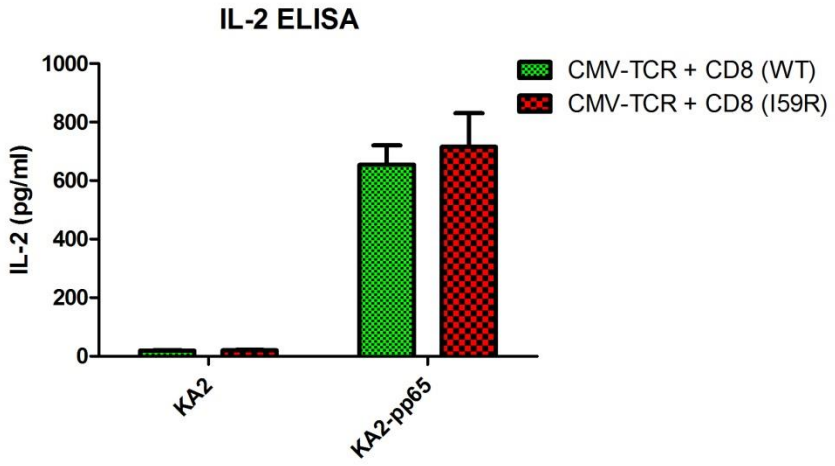


Figure 5-21 Recognition of KA2pp65 tumour cells expressing endogenously processed pp65 peptide induce IL-2 production by transduced CD4+ T cells was similar between CD8 wild-type and I59R mutant.
CD4+ T cells, transduced with CMV-TCR-CD8 vector containing CD8β wild-type (WT) or I59R mutation were incubated with KA2pp65 target or KA2 control tumour cells. IL-2 secretion was measured by ELISA. Shown is a representative result of at least two independent experiments from two different human donors.

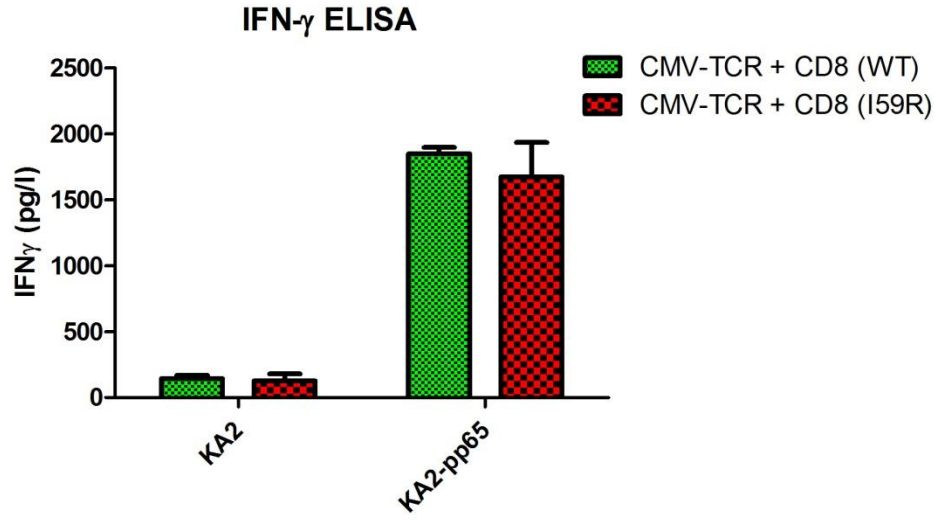


Figure 5-22 Recognition of KA2pp65 tumour cells expressing endogenously processed pp65 peptide induce IFN-γ production by transduced CD4+ T cells was similar between CD8 wild-type and I59R mutant.
CD4+ T cells, transduced with CMV-TCR-CD8 vector containing CD8β wild-type (WT) or I59R mutation were incubated with KA2pp65 target or KA2 control tumour cells. IFN-γ secretion was measured by ELISA. Shown is a representative result of at least two independent experiments from two different human donors.

Intracellular cytokine staining (ICS) was also performed to determine the proportion of CMV-TCR-td CD4+ T cells co-transduced with CD8 (WT) or CD8 (I59R) that produced Ag-specific responses to KA2pp65 or KA2 tumour cells (Fig 5-23). The set up was similar to the ELISA assay except that the cells were incubated for a shorter 6

hours before permeabilisation and fixation (Section 2.5.4). The transduced CD4+ T cells were identified by gating on CD8+ transduced CD4+ T cells. The CD4+ T cells transduced with CMV-TCR-CD8 (WT) compared to CMV-TCR-CD8 (I59R) vectors had similar levels of Ag-specific IL-2 (18.23% vs 17.13%) and IFN-γ (46.33% vs 44.01%) secretion.

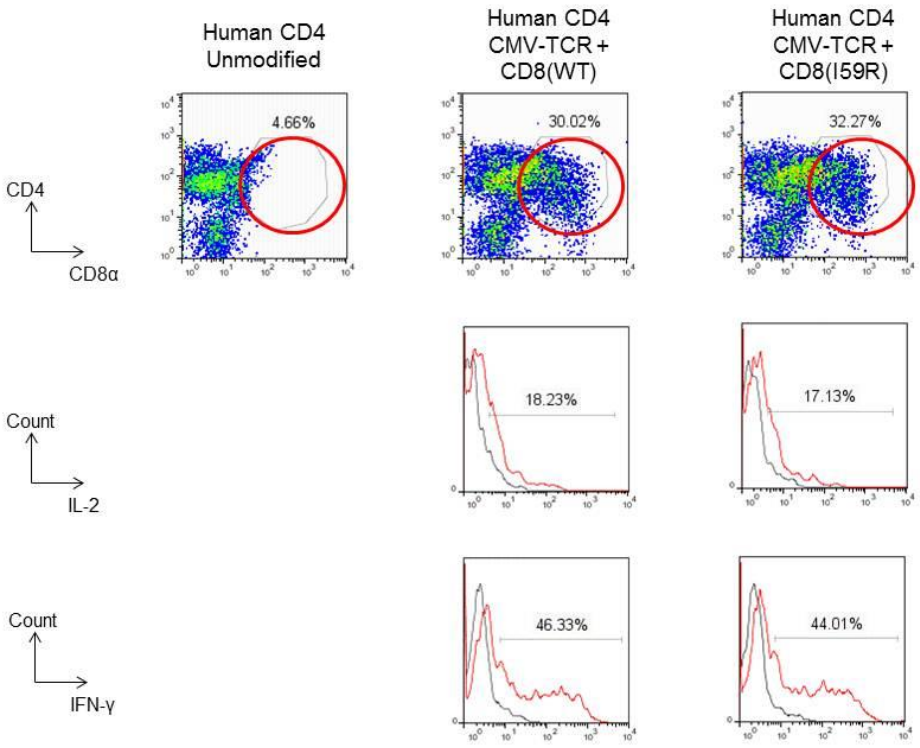


Figure 5-23 Recognition of KA2pp65 tumour cells induce IL-2 and IFN-γ production by transduced CD4+ T-cells was similar between CD8 wild-type and I59R mutant. Intracellular cytokine staining assay using human CD4+ T cells transduced with CMV-TCR-CD8 vector containing CD8β wild-type (WT) or I59R mutation following incubation with KA2pp65 target or KA2 control tumour cells for 6 hours. Transduced CD4+ T cells were gated (red circles) and analysed for IL-2 and IFN-γ production in respective columns. This result is representative of at least two independent experiments. This is a representative result of at least two independent experiments.

5.5 Summary

This chapter has described the effects of co-transfer of the wild-type CD8 co-receptor or CD8 co-receptors containing mutations into wild-type murine CD8+ T cells and CD4+ T cells. In the previous chapter, an augmenting CD8β L58R mutation and a diminishing CD8β TglyM were identified using functional assays performed with transduced CD8β-/- CD8+ T cells. These two mutations were chosen for further testing in wild-type CD8+ T cells and CD4+ T cells.

Perhaps it was not surprising that in F5-TCR transduced CD8+ T cells the introduction of CD8β L58R mutation did not further enhance Ag-specific IFN-γ or IL-2 production over CD8β wild-type. This was probably due to the presence of endogenous CD8 molecules minimizing the effects of additional CD8β co-receptor expression at the cell surface alongside the introduced TCR.

In F5-TCR transduced CD4+ T cells, the addition of the CD8 co-receptor significantly enhanced the Ag-specific IL-2 and IFN-γ production and this was further augmented by introducing the L58R mutation into the CD8 molecule. (Fig 5-10 to 5-14). The degree of augmentation in CD4+ T cells was much smaller than that observed in CD8β-/- CD8+ T cells and the degree of augmentation was only significant for the IL-2 cytokine after T cell stimulation with EL4NP tumour cells. In CD4+ T cells the expression of CD8β was limited by CD8α expression as transduction was performed using the CD8α-IRES-CD8β pMX vector. This was unlike the situation with the CD8β-/- T cells, with endogenous CD8α, in which the CD8β-pMP71 vector was used. The more obvious augmentation of IL-2 secretion compared to IFN-γ may be related to CD4+ T cells naturally favouring IL-2 to IFN-γ production. Lastly, the EL4NP cells were more potent at inducing Ag-specific responses than peptide loaded splenocytes, this may have been because of the greater surface expression of cognate antigen. The in-vivo effect of co-expression of CD8 (L58R) in TCR-transduced CD4+ T cells described in the following chapter (Chapter 6).

Lastly, utilizing the CD8 co-receptor with MHCI restricted TCR in human CD4+ T cells has also been explored by other members of the laboratory. Dr S Xue had showed that CMV-TCR and CD8 co-transduced CD4+ T cells function better than 'CMV-TCR alone' transduced CD4+ T cells. As the human and murine CD8 molecules are conserved the homologous mutation in the human CD8 co-receptor I59R was hypothesized to also be augmenting with respect to Ag-specific function. However experiments failed to show any enhancing properties using the CD8 I59R mutation (Fig 5-19 to Fig-23). This may be because the I59R mutation does not enhance MHCI binding due to differences between the human MHCI and the mouse MHCI, which were not explored here.

Chapter 6

Chapter 6. In-vivo work

6.1 Introduction

The in-vitro functional analysis of CD4+ T cells transduced with MHCI restricted F5-TCR demonstrated improved function after the CD8 co-receptor was introduced. The CD4+ T cells co-transduced with both F5-TCR and CD8 co-receptor produced both IL-2 and IFN-γ and had direct cytotoxic effects on EL4 tumour cells expressing the cognate peptide (EL4NP). Recent studies using transgenic murine CD4+ T cells (Xie et al. 2010; Quezada et al. 2010) and human CD4+ T cell clones (Hunder et al. 2008) have shown that adoptively transferred CD4+ T cells were able to eradicate tumour in-vivo through granzyme and IFN-γ production.

Although a few groups recently showed that CD4+ T cells transduced with TCR can eradicate tumours without requiring the addition of anti-tumour CD8+ T cells (Frankel et al. 2010; Kerkar et al. 2011; Soto et al. 2012), this required the use of a MHCII restricted TCR or a CD8 independent MHCI restricted TCR. In the situation where CD4+ T cells are transduced with a CD8 dependent TCR such as the F5-TCR, the addition of anti-tumour CD8+ T cells was required to induce tumour protection (Morris et al. 2005). As the CD8 co-receptor had augmented the in vitro cytokine production of F5-TCR transduced CD4+ T cells, the CD8 co-receptor may also augment the ability of F5-TCR co-transduced CD4 T cells to provide tumour protection.

6.2 Tumour protection with CD4+ T cells transduced with F5-TCR and CD8 co-receptor.

A schematic diagram of the in vivo experiment used is shown in Fig 6-1. To differentiate transferred T cells from endogenous T cells, transduced cells were taken from thy1.1+ C57Bl/6 mice and transferred into thy1.2 C57Bl/6 mice. Thy1.1 is found in T cells and commonly used as a congenic marker in cell transfer experiments. C57Bl/6 mice were sub-lethally irradiated (5.5 Gy) in order to improve engraftment and expansion of transferred thy1.1+ CD4+ T cells. Mice were injected with one million EL4NP tumour cells subcutaneously in the left flank. This dose of tumour cells was sufficient to induce 100% fatality in untreated mice (data from EL4NP tumour titration experiments performed by Dr E Nicholson). Mice challenged with EL4NP tumour were given different doses of CD4+ T cells transduced with F5-TCR and CD8 co-receptor by intravenous injection through a tail vein. This tested migration of transferred CD4+ T cells through the systemic circulation to the tumour site in the skin. Three T cell doses were chosen based on previous experiments from

$5x10^3$ to $5x10^5$ transduced CD4+ T cells. The control mice were given $5x10^5$ mock transduced CD4+ T cells. In this pilot study there were two mice per group. Tumour progression was monitored using direct measurement and when the tumour size was beyond 16mm or ulcerated the mice were culled. Surviving mice were examined for the persistence of the transferred CD4+ T cells using a congenic marker thy1.1.

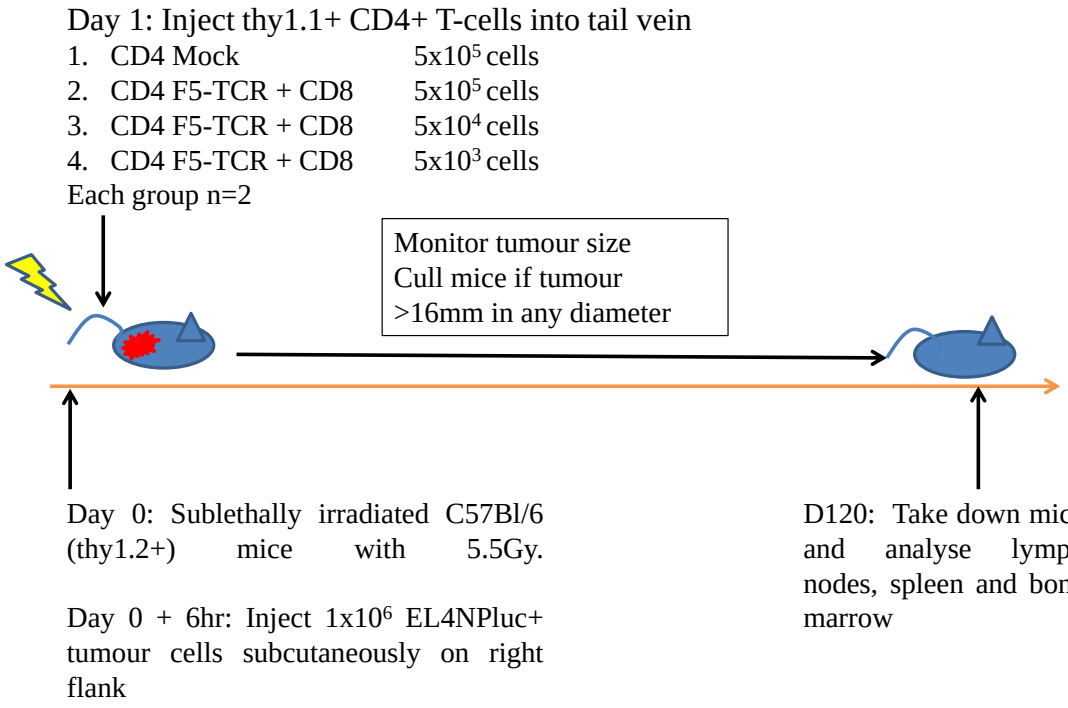


Figure 6-1 Schematic representation of the in-vivo model used to assess the ability of different doses of CD4+ T cells transduced with both F5-TCR and CD8 co-receptor to eradicate EL4NP tumour and persist.
Mice were sublethally irradiated (yellow sign) before injected with EL4NP tumour cells transduced with luciferase (EL4NPluc) followed by mock or different amounts of transduced CD4+ T cells into the tail vein. The time line is coloured orange.

6.2.1 Characterization of transferred transduced CD4+ T cells

Splenocytes taken from female thy1.1+ C57Bl/6 mice were enriched for CD4+ T cells then transduced with F5-TCR and CD8 molecules using separate vectors as described in Section 5.3.1. The population with high level CD8 co-receptor expression was enriched using anti-CD8α-APC antibodies and anti-APC microbeads. FACS analysis (Fig 6-2) of the CD8α CD4+ T cells following FACS sort showed enrichment of CD8+ CD4+ T cells, where >85% of CD4+ T cells expressed CD8α, with 38.6% of these also expressing Vβ11. The expression of CD8α molecules was proportional to the CD8β molecules, indicating heterodimer formation.

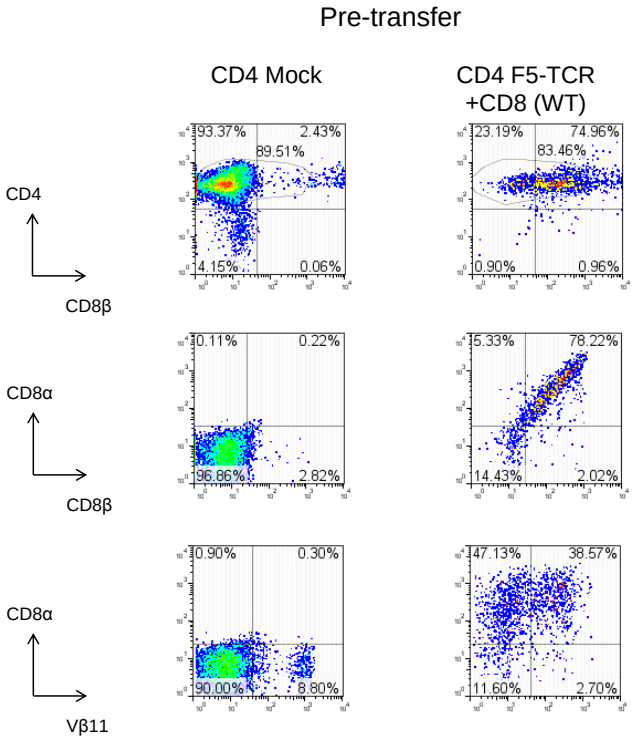


Figure 6-2 FACS analysis of CD8α sorted thy1.1+ CD4+ T cells transduced with F5-TCR and CD8 co-receptor.
Mock transduced CD4+ T cells were used for control. The first row show that the sorted live lymphocytes were predominantly CD4+ T cells which were gated (fine black line) and examined for expression of CD8 and TCR (Vβ11) on subsequent rows. Each column represents a separate condition.

6.2.2 Identification of adoptively transferred CD4+ T cells

Two weeks post T cell transfer, the persistence of transferred CD4+ T cells in the tumour bearing mice was confirmed by analysis of peripheral blood (Section 2.6.3.3). Transferred thy1.1+ CD4+ T cells were detected in all eight mice (Fig 6-3). At this time point, the transferred thy1.1+ transduced CD4+ T cells accounted for a greater proportion of live lymphocytes than thy1.1+ mock transduced CD4+ T cells (23.21% and 27.58% vs 5.84% and 4.59%) suggesting a potential survival advantage of transduced CD4+ T cells. The number of transduced CD4+ T cells found in the periphery at two weeks was also proportional to the dose transferred. When cell surface expression of the transduced CD8 and F5-TCR molecules were assessed in mice receiving the higher two doses of CD4+ T cells, down regulation of the CD8 co-receptor was observed with between 7.9% and 16.1% of the thy1.1+ CD4+ T cells still expressing the CD8 co-receptor. The F5-TCR expression using Vβ11 staining varied between 16.5% and 52.0%.

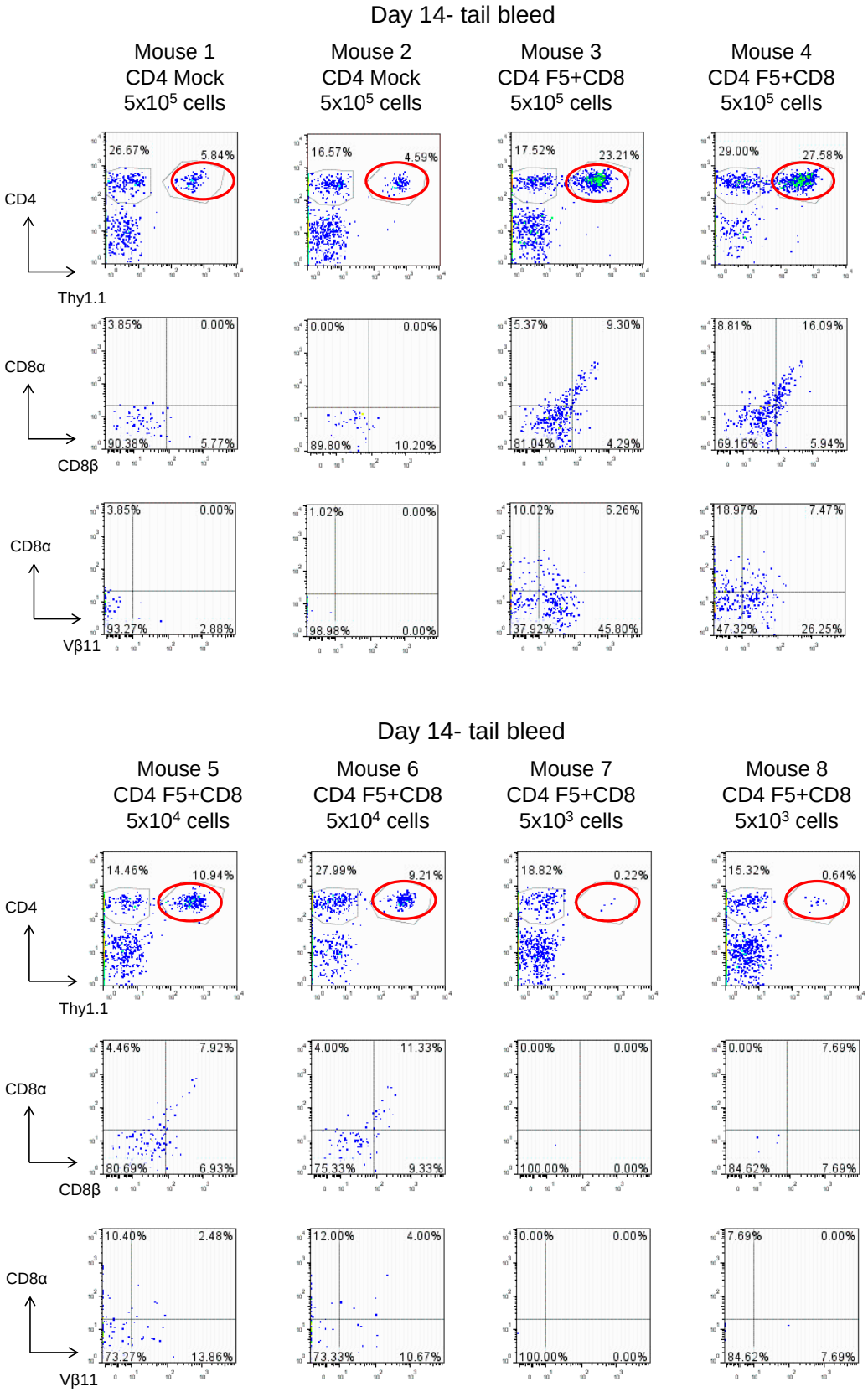


Figure 6-3 FACS analysis of peripheral bloood at D14 post transfer confirming the presence of transduced CD4+ T cells .
Mock transduced CD4+ T cells were used as control for gating purposes. The first row show the gating of transduced CD4+ T cells marked with thy1.1 (red circle) and examined for expression of CD8 and TCR (Vβ11) on subsequent rows. Each column represents a separate condition.

6.2.3 Tumour protection and survival

Tumour bearing mice were monitored at regular time intervals. The tumour mass was measurable from one week after injection. Mice receiving mock transduced CD4+ T cells had uncontrolled tumour growth with frequent ulceration. In addition, after two weeks these mice developed signs of distress and were culled. Mice receiving the lowest dose of transduced CD4+ T cells had uncontrolled tumour growth similar to mice receiving mock transduced CD4+ T cells (Fig 6-4). Mice receiving the highest dose of transduced CD4+ T cells had tumour protection evidenced by slower growth from day seven and subsequent tumour regression and undetectable tumour by day 38 (Fig 6-4). Of the two mice given the intermediate dose of transduced CD4+ T cells, one had uncontrolled tumour growth but the other developed significant tumour burden before tumour regression at a slower rate with undetectable tumour around day 42. Therefore a dose dependent effect was observed in mice receiving transduced CD4+ T cells where the highest dose ($5x10^5$ cells) resulted in rapid and sustained tumour control and the lower doses ($5x10^4$ and $5x10^3$) resulted in poorer tumour control and reduced survival (Fig 6-5). Of the three mice protected from tumour, no recurrence was observed even after 120 days of monitoring (Fig 6-5). Therefore the threshold for tumour protection may be around $5x10^4$ CD4+ T cells transduced with CD8 co-receptor and F5-TCR, which would need to be confirmed with experiments including larger groups of animals.

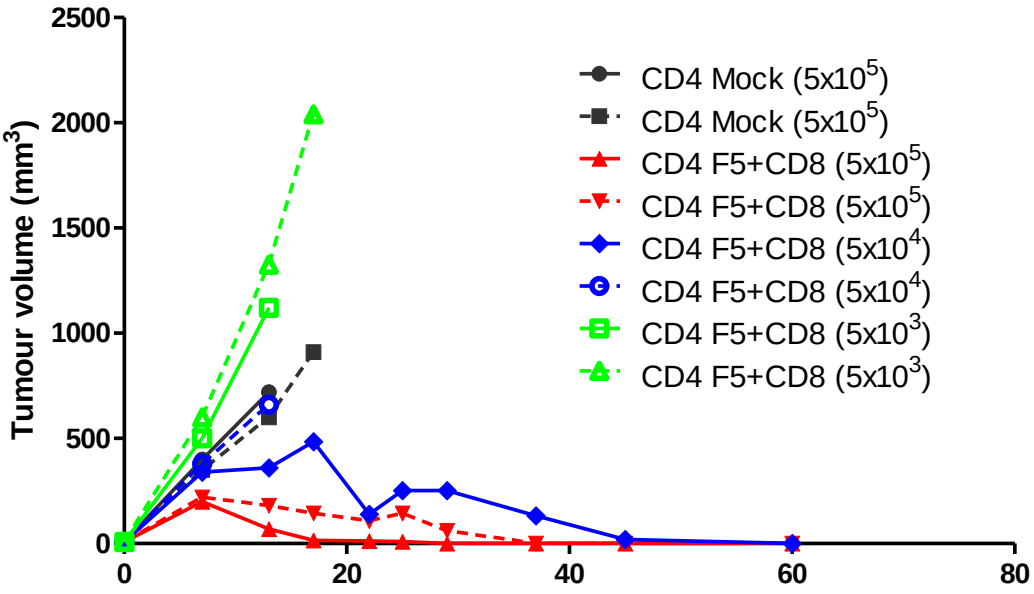


Figure 6-4 Titration experiment showing in vivo tumour protection is achieved by transferring as few as $5x10^4$ transduced CD4+ T cells into tumour bearing mice.
Different amounts of transduced CD4+ T cells shown in Section 6.2.1 were transferred into tumour bearing mice. Coloured lines representing shown indicate tumour volume recorded in individual mice at various time points (days).

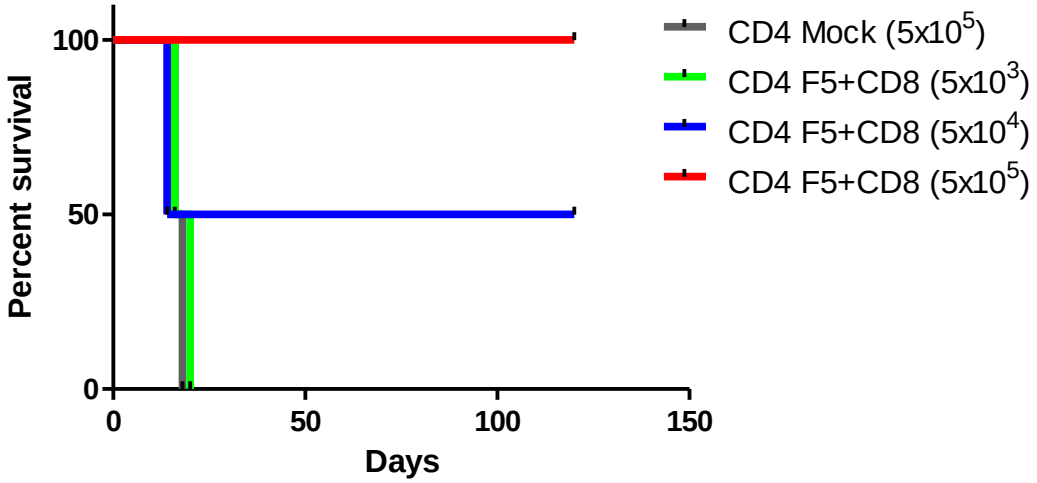


Figure 6-5 Survival curve showing in vivo tumour protection is achieved by transferring as few as 5x10^4 transduced CD4+ T cells into tumour bearing mice.
Different amounts of transduced CD4+ T cells shown in Section 6.2.1 were transferred into tumour bearing mice. Coloured lines show the percentage survival in each condition of two mice given the same amount of transduced CD4+ T cells.

6.2.4 Persistence of transferred CD4+ T cells in surviving mice

The three surviving mice were monitored for a total of 120 days after T cell transfer. After eradication of the primary tumour, the mice remained tumour-free for more than 60 days. To determine if transferred transduced CD4+ T cells persisted, the lymphoid organs spleen (spl), lymph node (LN) and bone marrow (BM) were examined at the end of the monitoring period. Thy1.1+ CD4+ T cells were found in all three lymphoid niches of all three mice (Fig 6-6 to 6-8). The two surviving mice given the highest dose of transduced CD4+ T cells had higher proportions of thy1.1 CD4+ T cells than the animal given the lower dose: spl 0.67%, 0.52% vs 0.09%, LN 0.33%, 0.41% vs 0.16%; BM 0.79%, 0.92% vs 0.09%.

To examine for cell surface expression of the transduced TCR and co-receptor together with the phenotype of persisting transduced cells, bulk cells taken from the lymphoid organs were stained for thy1.1, CD4, CD8α, CD8β, Vβ11, CD44 and CD62L. As there were no surviving mice given mock transduced thy1.1+ CD4+ T cells, the endogenous thy1.1neg CD4+ T cells were used to set the gates for the FACS analyses as shown in Fig 6-9 to 6-11. The majority of persisting transferred thy1.1+ CD4+ T cells expressed Vβ11+ suggesting a preferential survival advantage of F5-TCR transduced cells (Fig 6-6 to 6-8). There were two distinct Vβ11+ populations in the transferred thy1.1+ CD4+ T cells, the smaller Vβ11^{hi} population, which may be related to the small population of CD4+ T cells that constitutively express Vβ11 (ie, endogenous Vβ11+ cells in the transduced population) which was of a similar level to endogenous thy1.1neg Vβ11+ CD4+ or CD8+ T cells as shown in Figure 6-9 to 6-11. Significant down regulation of transduced CD8 co-receptor was observed with fewer

than 50% of the transferred thy1.1+ CD4+ T cells expressing CD8 at this time point. The level of expression of CD8 co-receptor on the transduced thy1.1+ CD4+ T cells was lower than the CD8 expression observed on endogenous thy1.1neg CD8+ CD4+ T cells. This was most evident from the intensity of CD8α staining in lymph node cells (MFI 88 vs 152).

Finally, the transferred thy1.1+ CD4+ T cells mostly (>80%) expressed the memory marker CD44 indicating exposure to antigen. The majority of persisting thy1.1+ CD4+ T cells had the effector memory CD44+ CD62Lneg phenotype (~70-80%) while the remainder were of the central memory CD44+ CD62L+ phenotype (Fig 6-6 to 6-8). The lymph nodes contained the greatest proportion of transferred CD4+ T cells with the central memory phenotype (26%-37%).

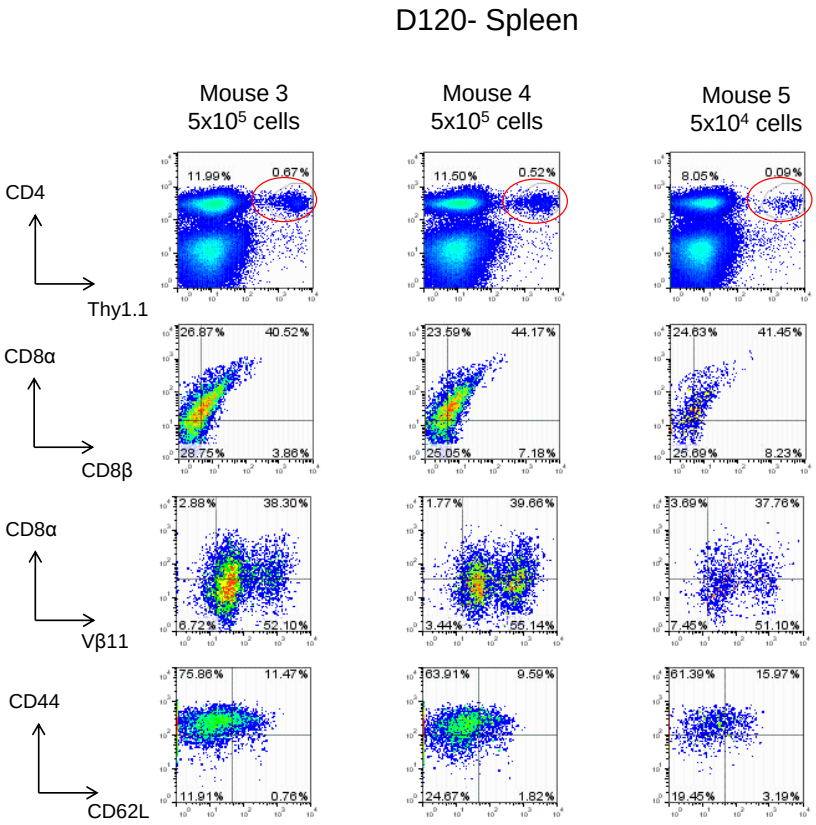


Figure 6-6 Ex vivo phenotypic analysis of CD4+ thy1.1+ (transferred) cells taken from spleen 120 days after adoptive transfer.
The first row show the gating of transduced CD4+ T cells marked with thy1.1 (red circle) and examined for expression of CD8 and TCR (Vβ11) as well as memory markers CD44 and CD62L on subsequent rows. Each column represents a separate mouse.

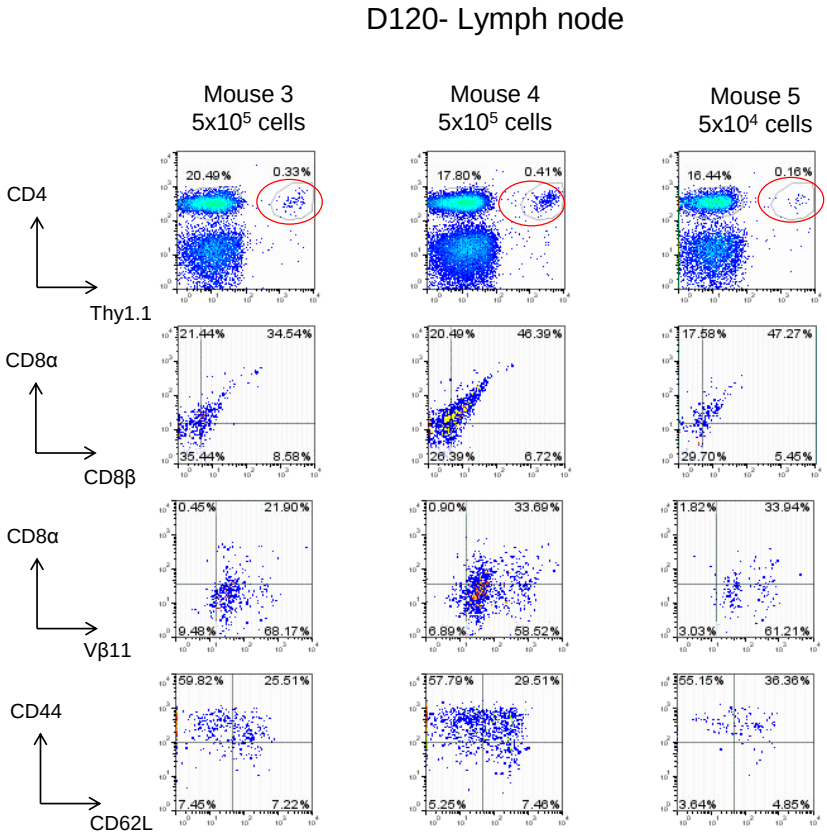


Figure 6-7 Ex vivo phenotypic analysis of CD4+ thy1.1+ (transferred) cells taken from lymph nodes 120 days after transfer.
The first row show the gating of transduced CD4+ T cells marked with thy1.1 (red circle) and examined for expression of CD8 and TCR (Vβ11) as well as memory markers CD44 and CD62L on subsequent rows. Each column represents a separate mouse.

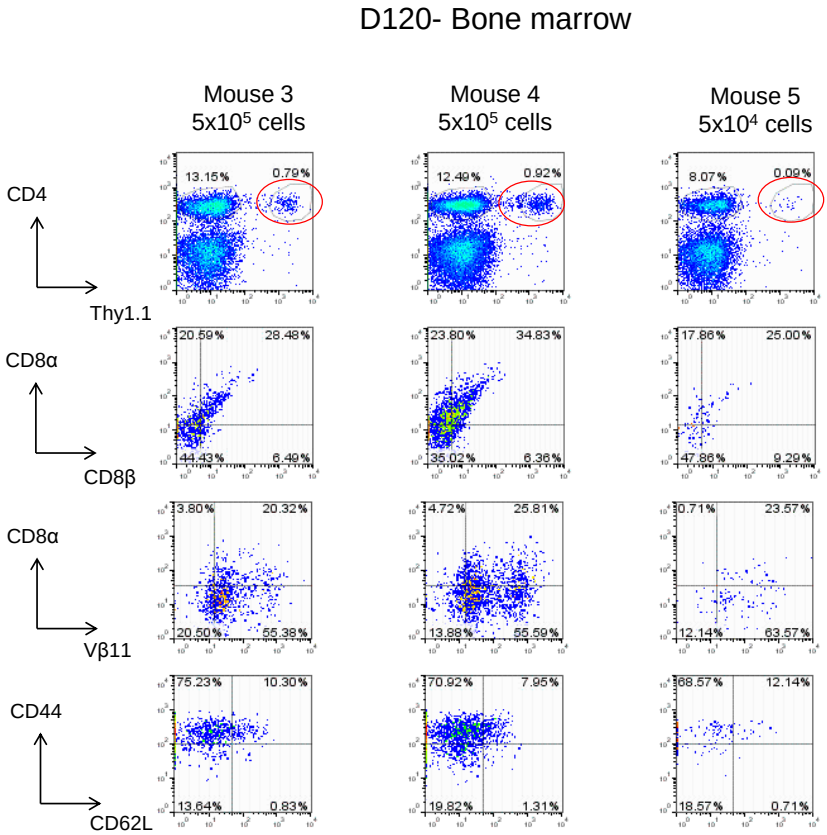


Figure 6-8 Ex vivo phenotypic analysis of CD4+ thy1.1+ (transferred) cells taken from bone marrow 120 days after transfer.
The first row show the gating of transduced CD4+ T cells marked with thy1.1 (red circle) and examined for expression of CD8 and TCR (Vβ11) as well as memory markers CD44 and CD62L on subsequent rows. Each column represents a separate mouse.

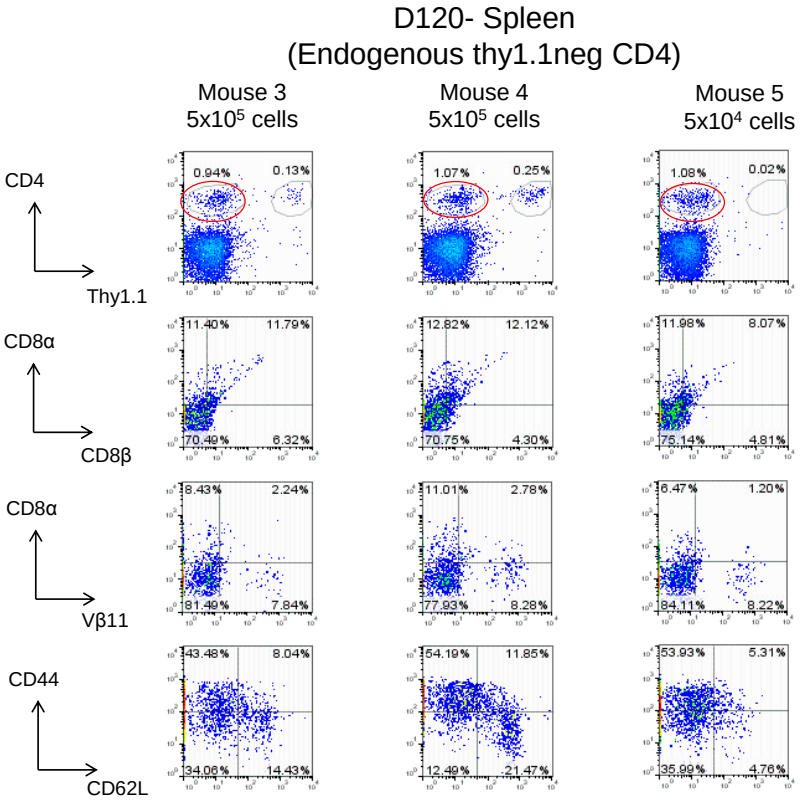


Figure 6-9 Ex vivo phenotypic analysis of CD4+ thy1.1neg (endogenous) cells taken from spleen 120 days after transfer.
The first row show the gating of endogenous CD4+ T cells that were thy1.1neg (red circle) and examined for expression of CD8 and TCR (Vβ11) as well as memory markers CD44 and CD62L on subsequent rows. Each column represents a separate mouse.

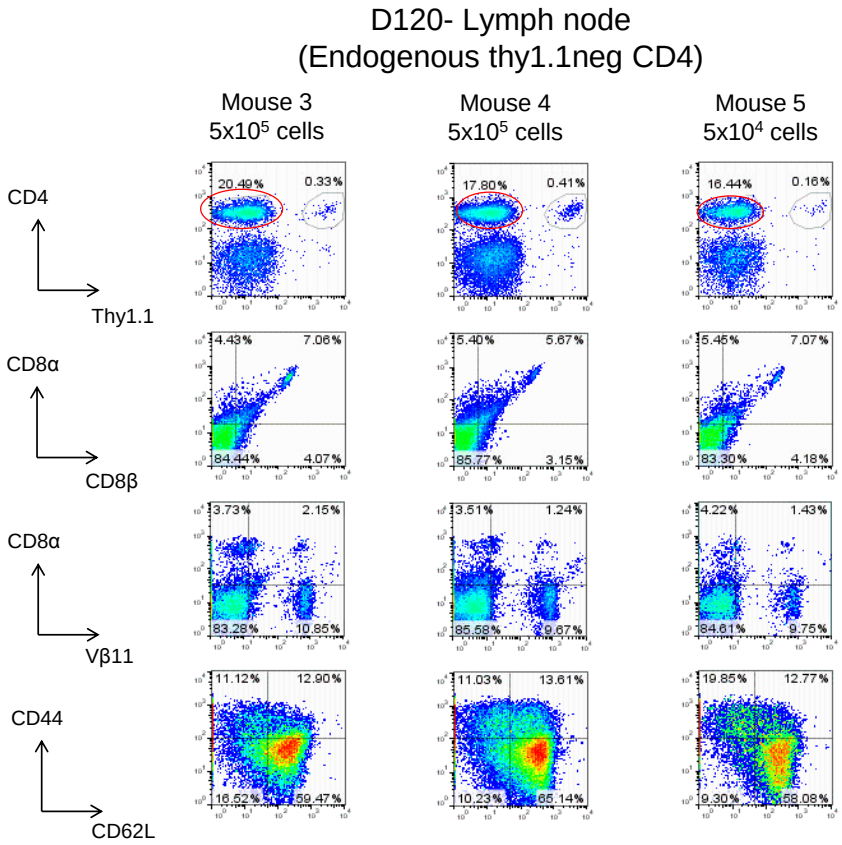


Figure 6-10 Ex vivo phenotypic analysis of CD4+ thy1.1neg (endogenous) cells taken from lymph nodes 120 days after transfer.
The first row show the gating of endogenous CD4+ T cells that were thy1.1neg (red circle) and examined for expression of CD8 and TCR (Vβ11) as well as memory markers CD44 and CD62L on subsequent rows. Each column represents a separate mouse.

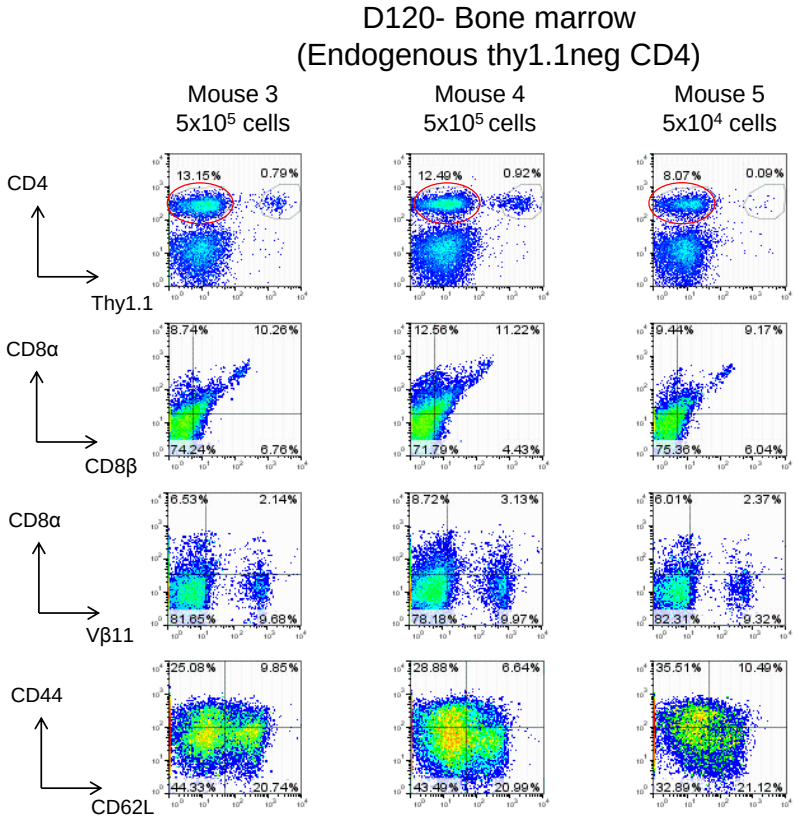


Figure 6-11 Ex vivo phenotypic analysis of CD4+ thy1.1neg (endogenous) cells taken from bone marrow 120 days after transfer.
The first row show the gating of endogenous CD4+ T cells that were thy1.1neg (red circle) and examined for expression of CD8 and TCR (Vβ11) as well as memory markers CD44 and CD62L on subsequent rows. Each column represents a separate mouse.

6.3 *In vivo tumour protection following adoptive transfer of transduced CD4+ T cells*

With the knowledge that transduced CD4+ T cells have the potential to eradicate tumour in an antigen specific manner and persist indefinitely, the subsequent in-vivo experiment was designed to compare F5-TCR transduced CD4+ T cells with or without CD8 co-receptor and also to compare the effects of CD8 co-receptor containing CD8β wild-type (WT) or CD8β containing the L58R mutation. The model used to study this is shown in a schematic diagram (Fig 6-12). As in the preliminary in vivo experiment described above, C57Bl/6 mice were sublethally irradiated with 5.5 Gy in order to enhance engraftment and expansion of transferred CD4+ T cells. Mice were injected with one million EL4NP tumour cells expressing luciferase (EL4luc) subcutaneously in the left flank. Mice challenged with EL4NP tumour were given $1x10^5$ F5-TCR transduced CD4+ T cells with or without CD8 co-receptor by intravenous injection through a tail vein. This sub-therapeutic dose of transduced CD4+ T cells was chosen in order to compare the efficacy of CD8 co-receptor

containing the CD8β WT or CD8β L58R. The control mice received tumour followed by $1x10^5$ mock transduced CD4+ T cells. Tumour progression was monitored using direct measurement and detection of bioluminescence. When the tumour size was beyond 16mm or had ulcerated mice were culled. Surviving mice were re-challenged with irradiated EL4NP tumour cells and then culled five days later in order to study recall responses. Each of the two in-vivo tumour protection experiments had a total of 18 mice (ie, 36 mice in total). Each group of mice were housed in the same cage and ear tagged for identification.

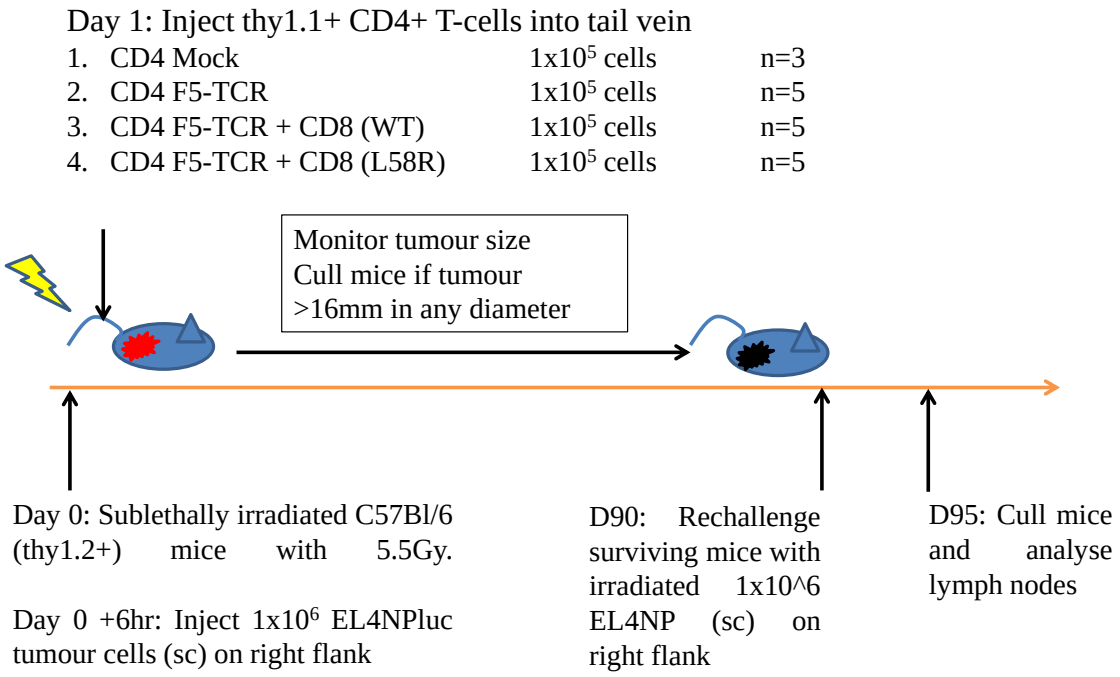


Figure 6-12 Schematic representation of the experimental model used to compare the ability of F5-TCR transduced CD4+ T cells with or without CD8 co-receptor to eradicate EL4NP tumour, persist and respond to Ag re-challenge.
Mice were sublethally irradiated (yellow sign) before injected with EL4NP tumour cells transduced with luciferase (EL4NPluc) followed by mock or different types of transduced CD4+ T cells into the tail vein. The time line is coloured orange. Two independent experiments were performed.

6.3.1 Characterization of transferred transduced CD4+ T cells

Splenocytes taken from female thy1.1+ C57Bl/6 mice were enriched for CD4+ T cells and transduced with F5-TCR alone or with the CD8 co-receptor containing CD8β WT or CD8β L58R using separate vectors as described in Section 5.3.1. The CD8 transduced populations were enriched using anti-CD8α-APC antibodies and anti-APC microbeads. FACS analysis of CD4+ T cells (Fig 6-13) post sorting showed enrichment of CD8+ CD4+ T cells of (>80% of CD4+ T cells) and enrichment for Vβ11 expression in the CD8 WT and CD8 L58R transduced populations (both 51%).

The expression of CD8α was proportional to CD8β expression indicating appropriate heterodimer formation at the cell surface. Of the adoptively transferred transduced CD8+ CD4+ T cells about half of the cells expressed the F5-TCR and were able to recognize tumour.

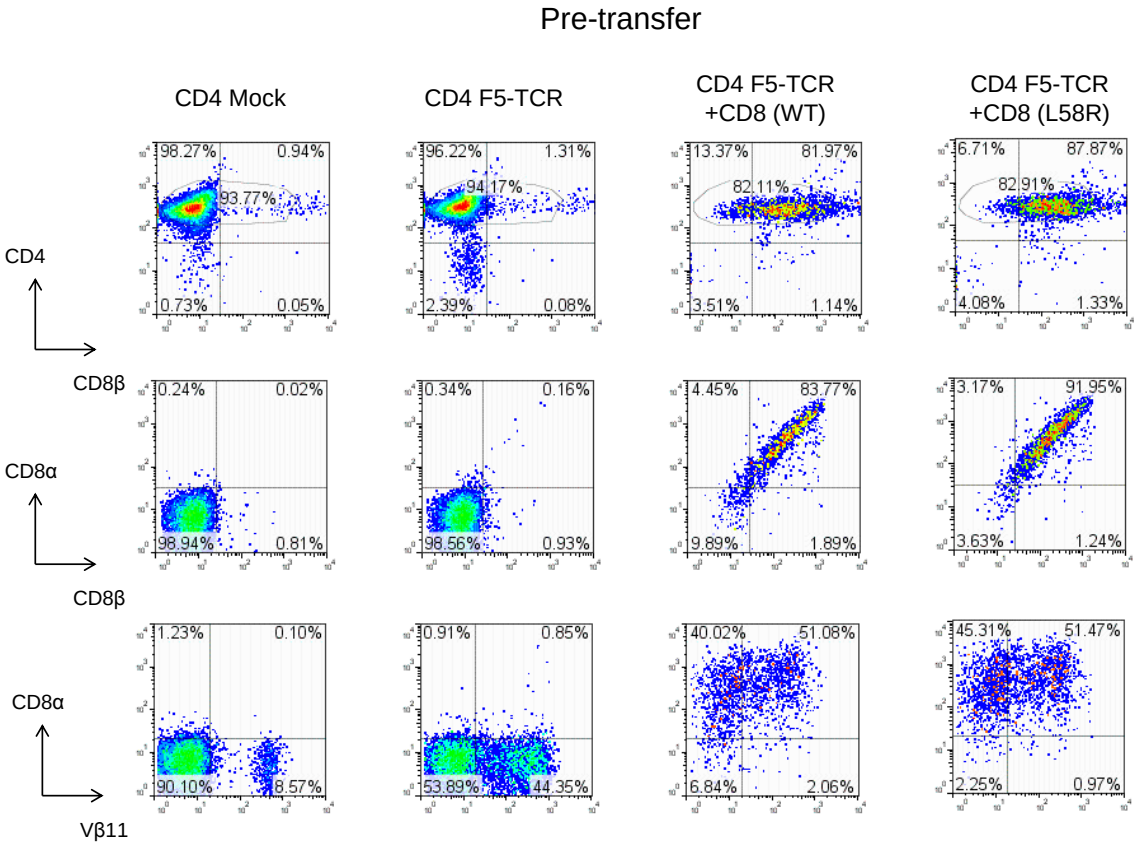


Figure 6-13 FACS analysis of CD8α sorted thy1.1+ CD4+ T cells transduced with F5-TCR alone or with CD8 co-receptor containing CD8β WT or CD8β L58R.
Mock transduced CD4+ T cells were used for control. The first row show that the sorted live lymphocytes were predominantly CD4+ T cells which were gated (fine black line) and examined for expression of CD8 and TCR (Vβ11) on subsequent rows. Each column represents a separate condition.

6.3.2 Identification of transferred cells

Two weeks post cell transfer, in vivo persistence of transferred thy1.1+ CD4+ T cells was confirmed by analysis of peripheral blood (Section 2.6.3.3). The number of transduced thy1.1+ CD4+ T cells identified in the periphery at two weeks was similar in all the conditions, range 1-10% (Fig 6-14 to 6-17). Significant down regulation of the CD8 co-receptor was seen previously in the CD4+ T cells transduced with the CD8 co-receptor. The proportion of cells in the periphery still expressing CD8 co-receptor was significantly lower in the CD8 WT condition than in the CD8 L58R condition (8.5% vs 22.5%; p=0.0252). The F5-TCR expression using Vβ11 antibodies range from 27% to 89%.

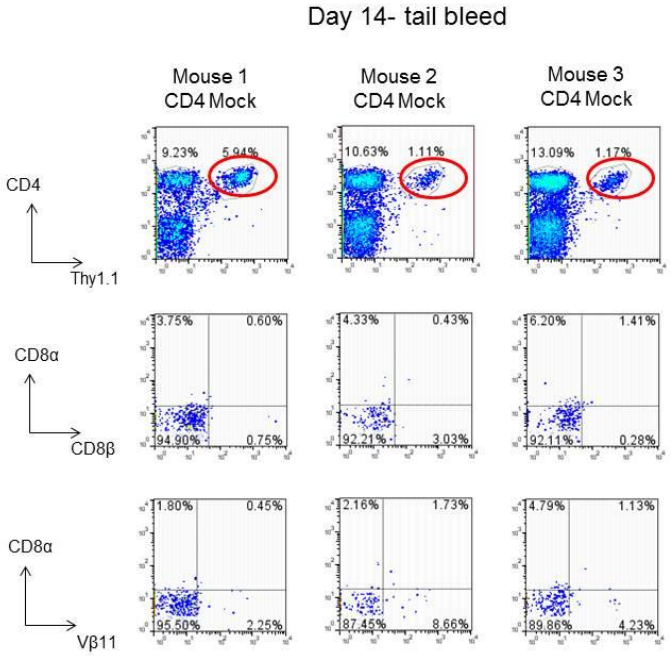


Figure 6-14 FACS analysis of peripheral bloood at D14 post transfer confirming the presence of mock transduced CD4+ T cells in all three mice.
Mock transduced CD4+ T cells were used as control for gating purposes. The first row show the gating of transduced CD4+ T cells marked with thy1.1 (red circle) and examined for expression of CD8 and TCR (Vβ11) on subsequent rows. Each column represents a separate condition.

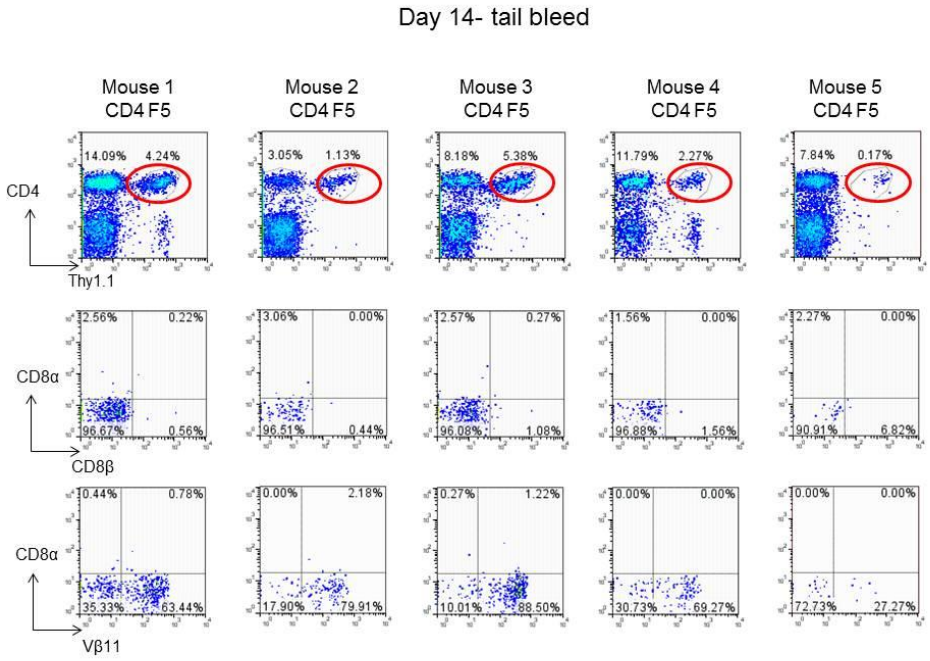


Figure 6-15 FACS analysis of peripheral bloood at D14 post transfer confirming the presence of F5-TCR transduced CD4+ T cells in all five mice.
Mock transduced CD4+ T cells were used as control for gating purposes. The first row show the gating of transduced CD4+ T cells marked with thy1.1 (red circle) and examined for expression of CD8 and TCR (Vβ11) on subsequent rows. Each column represents a separate condition.

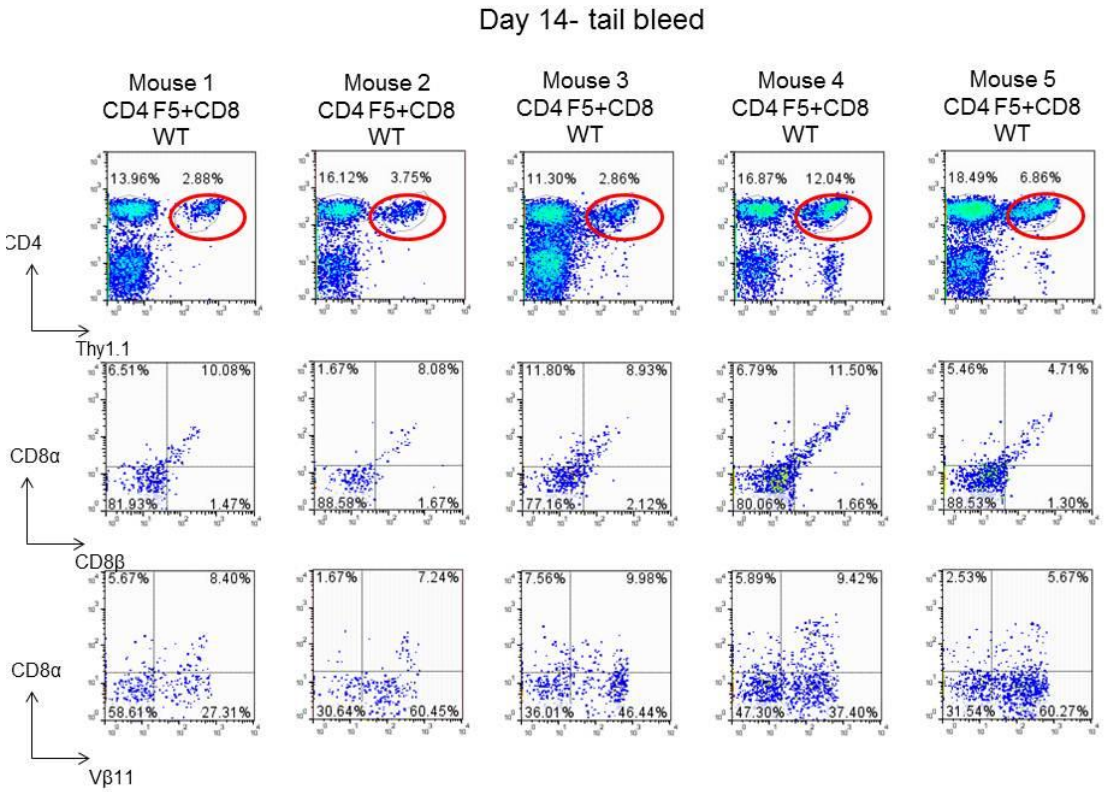


Figure 6-16 FACS analysis of peripheral bloood at D14 post transfer confirming the presence of F5-TCR and CD8 co-transduced CD4+ T cells in all five mice.
Mock transduced CD4+ T cells were used as control for gating purposes. The first row show the gating of transduced CD4+ T cells marked with thy1.1 (red circle) and examined for expression of CD8 and TCR (Vβ11) on subsequent rows. Each column represents a separate condition.

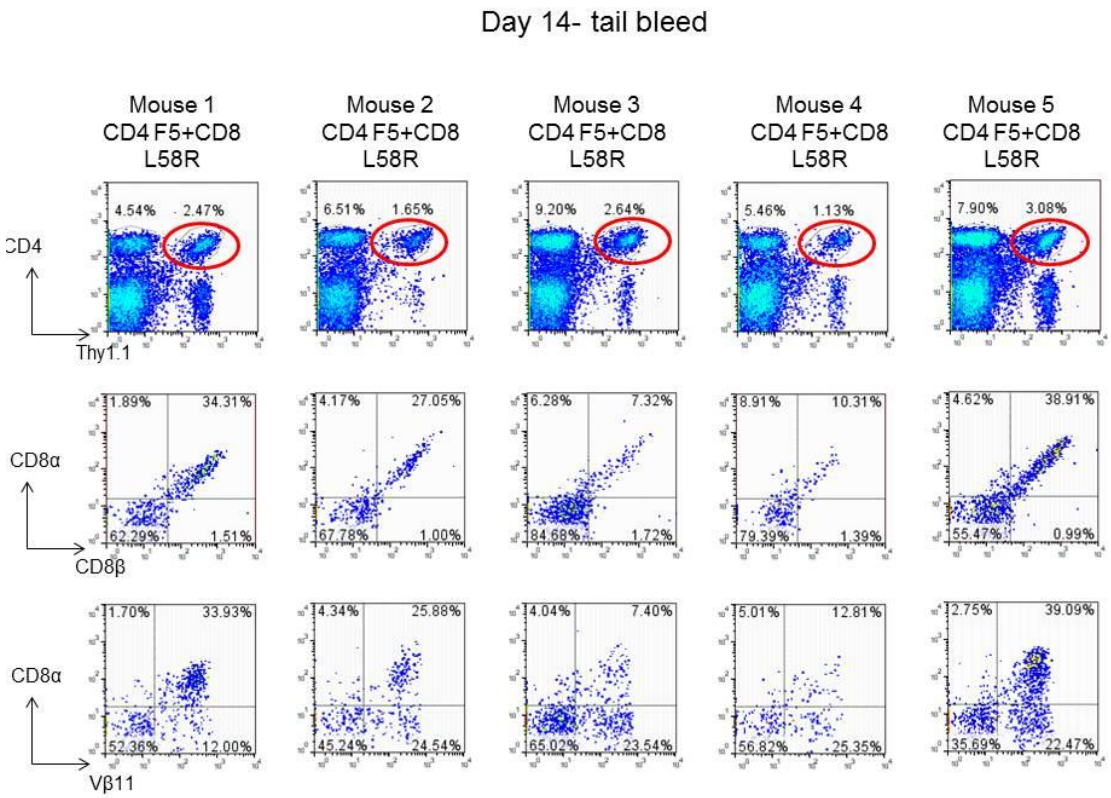


Figure 6-17 FACS analysis of peripheral bloood at D14 post transfer confirming the presence of mock transduced CD4+ T cells in all three mice.
Mock transduced CD4+ T cells were used as control for gating purposes. The first row show the gating of transduced CD4+ T cells marked with thy1.1 (red circle) and examined for expression of CD8 and TCR (Vβ11) on subsequent rows. Each column represents a separate condition.

6.3.3 Effect of adoptively transferred transduced CD4+ T cells on tumour protection and survival

Tumour bearing mice were monitored at regular time intervals as described above. The tumour became evident and could be measured from one week by direct measurement (Fig 6-18 and 6-19) or by bioluminescence. The bioluminescence produced by EL4NPluc could be visualized through pseudo-colouring of intensity or by quantification the photons/s produced from each individual mouse (Fig 6-20 and 6-21). Mice receiving mock transduced CD4+ T cells had uncontrolled tumour growth (Fig 6-18 and 6-20) resulting in ulceration with central necrotic plaques which artificially reduced bioluminescence readings. After two weeks even when tumour growth diameter did not exceed limits these mice became distressed and were culled. Mice receiving F5-TCR-td CD4+ T cells had uncontrolled tumour growth resulting in large tumours but the condition of the mice was better than those receiving CD4 mock transduced CD4+ T cells (Fig 6-18, 6-20 and 6-21). Only 2 out of the 10 mice given F5-TCR transduced CD4+ were protected and survived beyond one month.

Mice receiving CD4+ T cells transduced with F5-TCR and CD8 co-receptor had slower tumour growth rate between day 7 and day 14 before tumour shrinkage from around day 21 and eventual tumour eradication. This was evident from both direct tumour measurements (Fig 6-18 and 6-19) as well as bioluminescence measurements (Fig 6-20 and 6-21). Mice receiving CD4+ T cells transduced with the CD8 L58R beta chain co-receptor had marginally enhanced tumour protection. The average tumour volume at day 21 in the CD8 L58R treated mice was smaller than in the CD8 WT treated mice ($491mm^3$ vs $882mm^3$; p=0.104) as shown in Figure 6-19. In a few mice, two in the CD8 WT condition and one in the CD8 L58R condition, there was recurrence of tumour after some tumour shrinkage (Fig 6-18). The tumour recurrence may have been due to antigen loss resulting in tumour escape as one mice examined had thy1.1+ CD4+ T cells that produced IL-2 and IFN-γ to fresh EL4NP tumour cells but not to EL4NP tumour cells isolated from the recurrent tumour. This has recently been described by our laboratory during the use of F5-TCR transduced CD8+ T cells in same EL4NP tumour model (Velica 2012).

Data was combined from the two independent experiments to generate a survival curve. The survival of mice given F5-TCR transduced CD4+ T cells was poor with only 2/10 surviving. Co-transducing CD8 co-receptor (WT) improved the survival rate to 5/10 and co-transducing CD8 containing the L58R mutation further improved the survival rate to 8/10 (Fig 6-21).

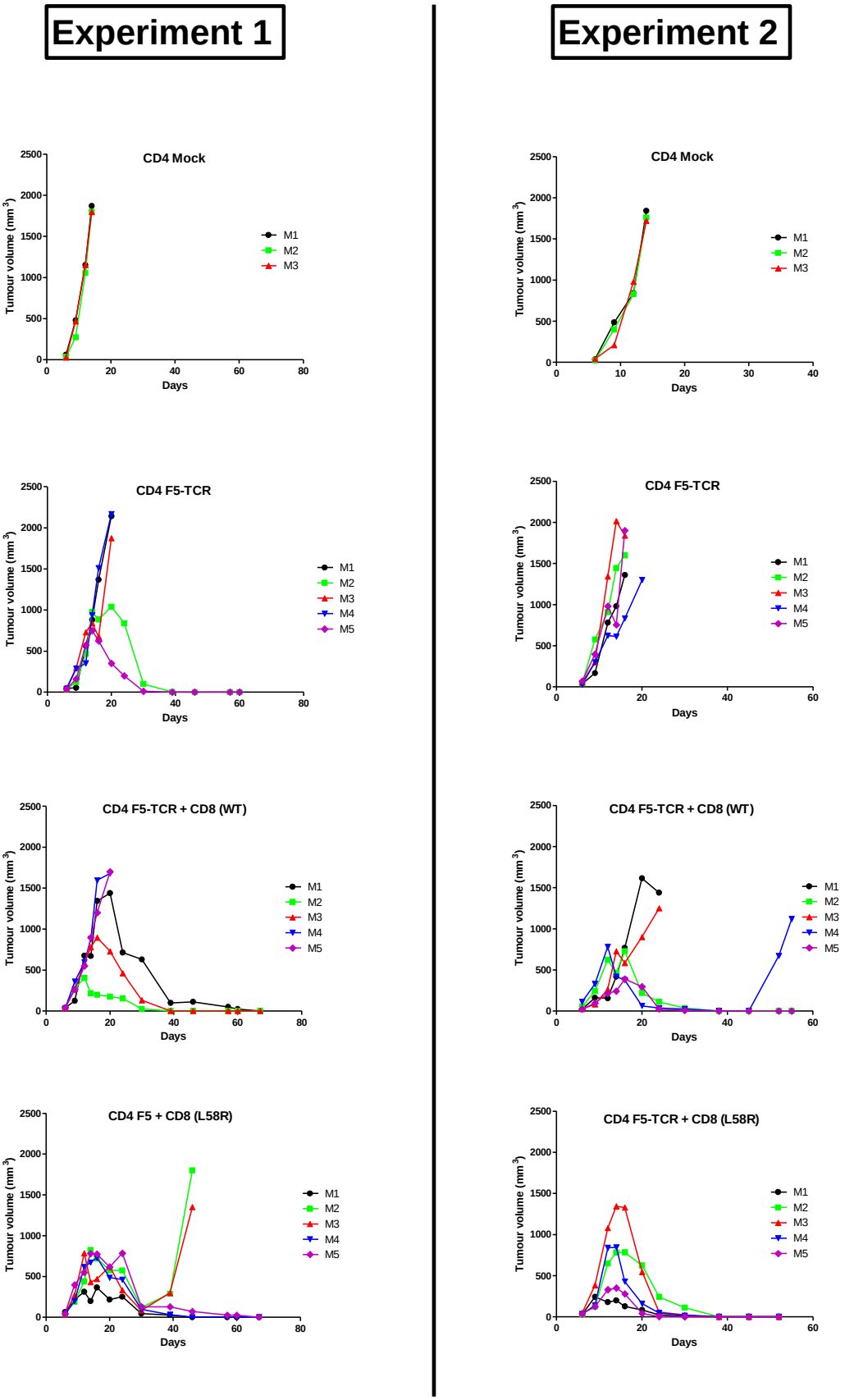


Figure 6-18 In vivo tumour protection was superior when F5-TCR/CD8 CD4+ T cells compared to F5-TCR CD4+T cells was transferred into tumour bearing mice.
The results of two independent experiments are shown here with the first (left) and the second (right) separated by a vertical line. Each condition received the same $1x10^5$ numbers of mock or transduced CD4+ T cells shown in Section 6.3.1. Coloured lines representing shown indicate tumour volume recorded in individual mice at various time points (days).

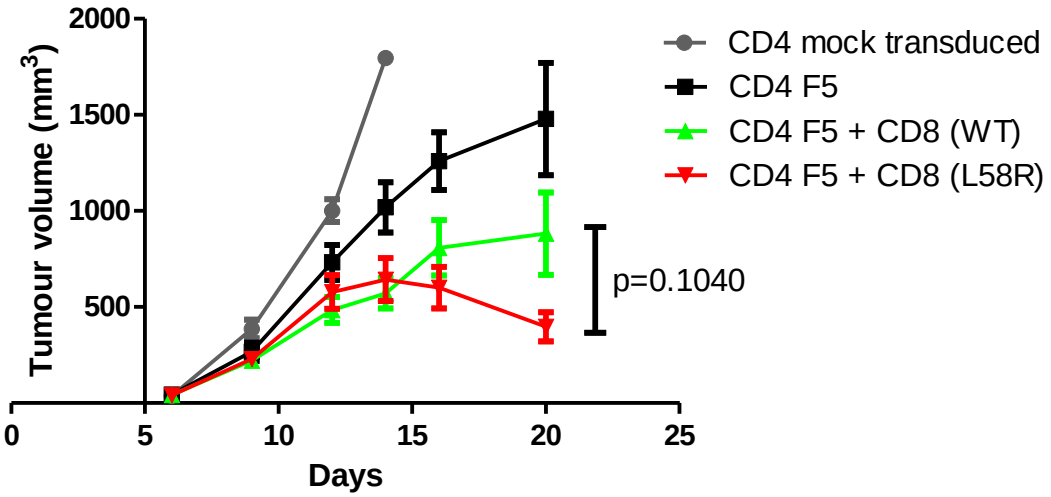


Figure 6-19 Control of tumour growth after transfer of F5-TCR transduced CD4+ T cells show superior early tumour control when CD8 coreceptor is co-transduced.
Mean tumour volume measured from day 6 after CD4+T cell transfer. This is pooled data from two independent experiments (n=6 for CD4 mock and n=10 for the F5 TCR-td groups).

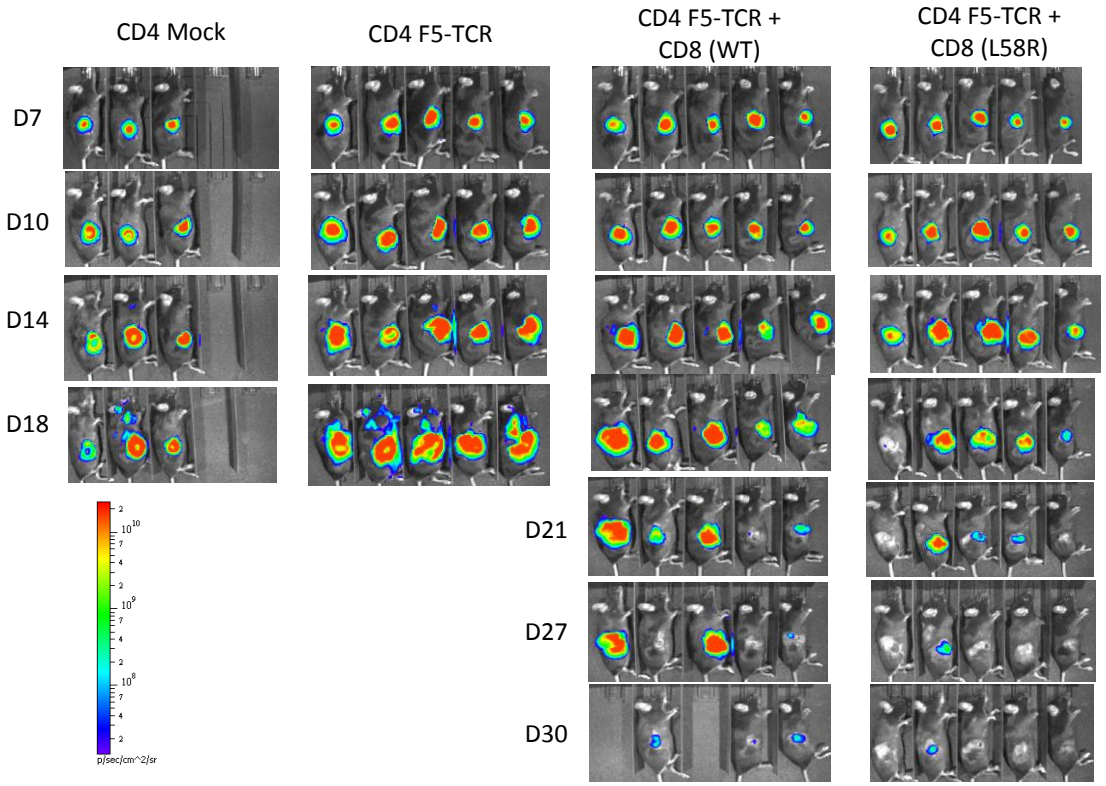


Figure 6-20 CD4+ T cells transduced with F5-TCR and CD8 co-receptor reduce tumour burden visualised using bioluminescence.
Visualisation of bioluminescence from EL4NPluc tumour in mice after injection of D-luciferin and shown at the indicated time points. Each individual mice from each condition is lined up in columns. The bioluminescence is processed using living image 3.2 software and expressed in a logarithmic colour scale. One of two independent experiments is shown.

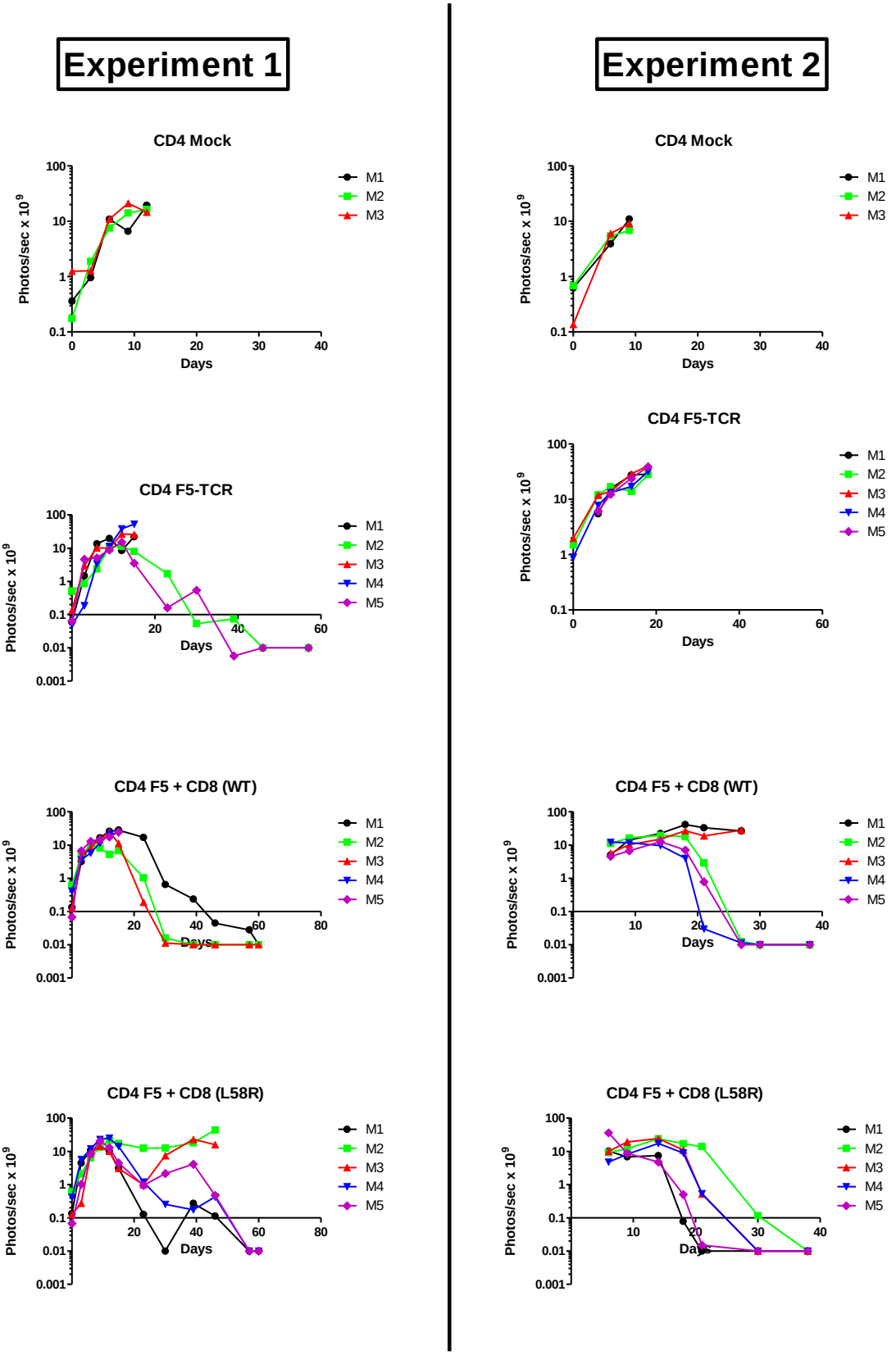


Figure 6-21 CD4+ T cells co-transduced with F5-TCR and CD8 co-receptor reduces tumour burden based on objective measures using bioluminescence.
Tumour growth of individual tumour bearing mice from both in-vivo experiments was monitored by IVIS-100 bioluminescence camera at the indicated time points. The graph shows the bioluminescence signals of individual mice in photons/s expressed on a logarithmic scale.

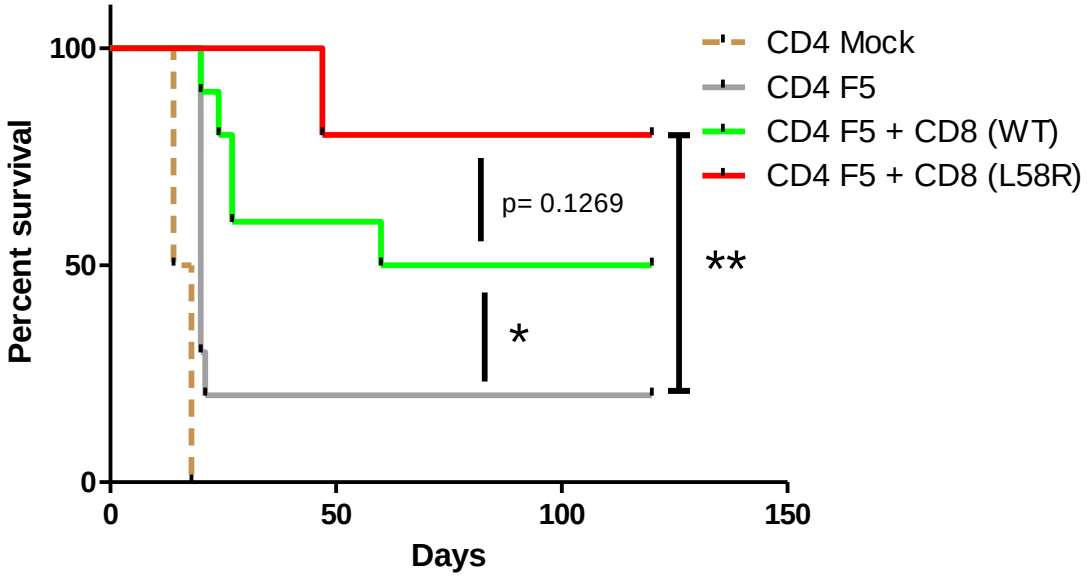


Figure 6-22 CD4+ T cells transduced with F5-TCR and CD8 co-receptor improve survival of tumour bearing mice.
Combined results of two independent experiments n=6 for CD4 mock and n=10 for the F5 TCR-td groups. For survival analysis curves were analysed by the log-rank test; *, $P < 0.05$; ** $P < 0.005$.

6.3.4 In vivo Ag-specific re-call responses of adoptively transferred transduced CD4+ T cells

The in vivo persistence of transferred CD4+ T cells for prolonged periods provided an opportunity to examine the Ag-specific secondary responses against EL4NP tumour. It was noted from the earlier preliminary experiment in section 6.2 that the mouse given sub-therapeutic $0.5x10^5$ CD4+ T cells had low numbers of persisting cells in the lymph node, spleen and bone marrow even at 120 days post introduction (Fig 6-6 to 6-8). To determine if the small numbers of persisting transduced CD4+ T cells could proliferate on re-encounter with antigen, irradiated EL4NP was injected subcutaneously into the right lower leg of the surviving mice from the subsequent in-vivo experiment (described above in Section 6.3.3). There were different numbers of surviving mice from the different groups; the F5-TCR alone group had only two surviving mice, the F5-TCR and CD8 (WT) group had five surviving mice and the F5-TCR and CD8 (L58R) group had eight surviving mice. Five days after re-challenge, the mice were culled and lymph nodes analysed for the presence of transferred thy1.1+ CD4+ T cells.

The lymph nodes from the right popliteal and inguinal LN which were the tumour draining lymph nodes (TDLN) and the left popliteal and inguinal LN which were the non-draining lymph nodes (NDLN) were analyzed separately. FACS analysis demonstrated the persistence of transferred thy1.1+ CD4+ T cells, with the majority (>50%) of cells expressing Vβ11 (Fig 6-23 and 6-24). Enumeration of the transferred thy1.1+ CD4+ from each set of lymph nodes showed that a greater absolute number

of thy1.1+ CD4+ T cells were detected in the TDLN than in the NDLN consistent with secondary expansion (Fig 6-25). However, the tumour induced proliferation of thy1.1+ CD4+ T cells was not significantly different between the different groups of transferred thy1.1+ CD4+ T cells (Fig 6-25). In the mice given CD4+ T cells with CD8 co-receptor, most of this increase was accounted by CD8+ CD4+ T cells (Fig 6-26) but no significant difference was observed between CD4+ T cells transduced with CD8 WT or CD8 L58R.

When the memory phenotype of the CD8neg or CD8+ transferred thy1.1+ CD4+ T cells from TDLN were compared, it was demonstrated that the CD8+ T cells were skewed to a central memory (CD44+ CD62+) phenotype rather than an effector (CD44+ CD62neg) phenotype (Fig 6-27 and 6-28).

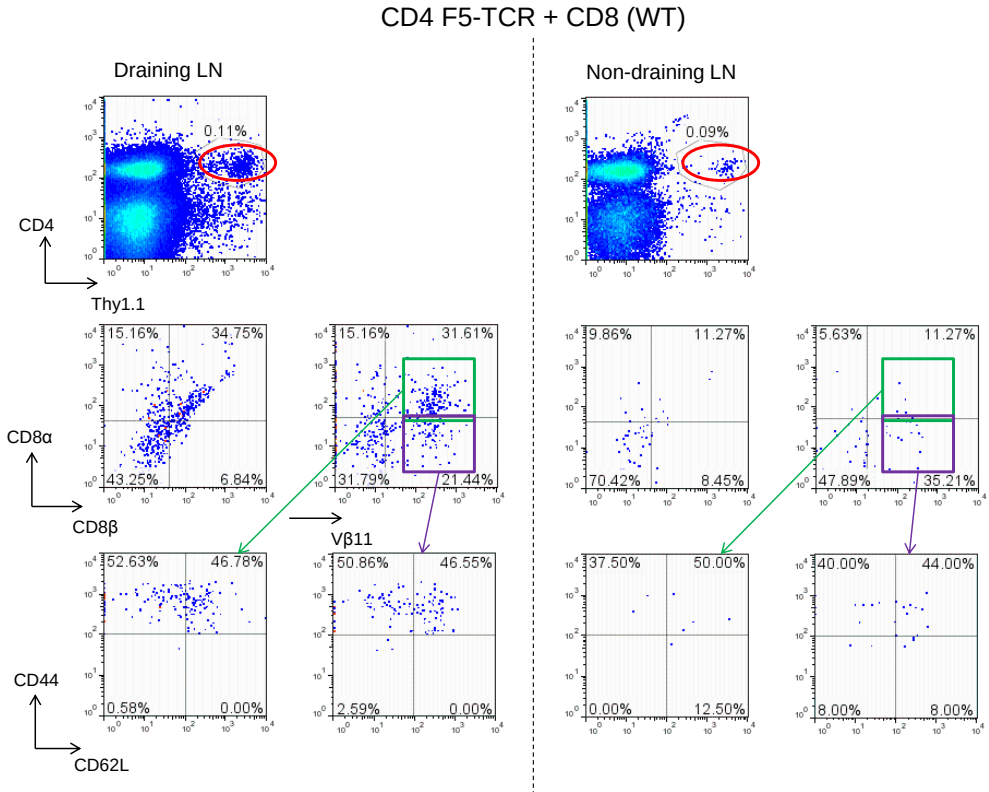


Figure 6-23 Ex vivo phenotypic analysis of lymphoid cells taken from draining (TDLN) with non-draining (NDLN) lymph nodes after Ag re-challenge in mice previously treated with thy1.1+ CD4+ CD8(WT) F5-TCR transduced T cells.
Example FACS plots shown from one surviving animal. Endogenous thy1.1neg CD4+ T cells were used as controls for gating purposes. The F5-TCR was detected by anti-Vβ11 antibodies. The cells shown were first gated on live lymphocytes and then thy1.1+ CD4+ T cells (red circle). CD8α+ (green square) and CD8α- (purple square) Vβ11+ T cells were examined separately for memory phenotype using CD44 and CD62L.

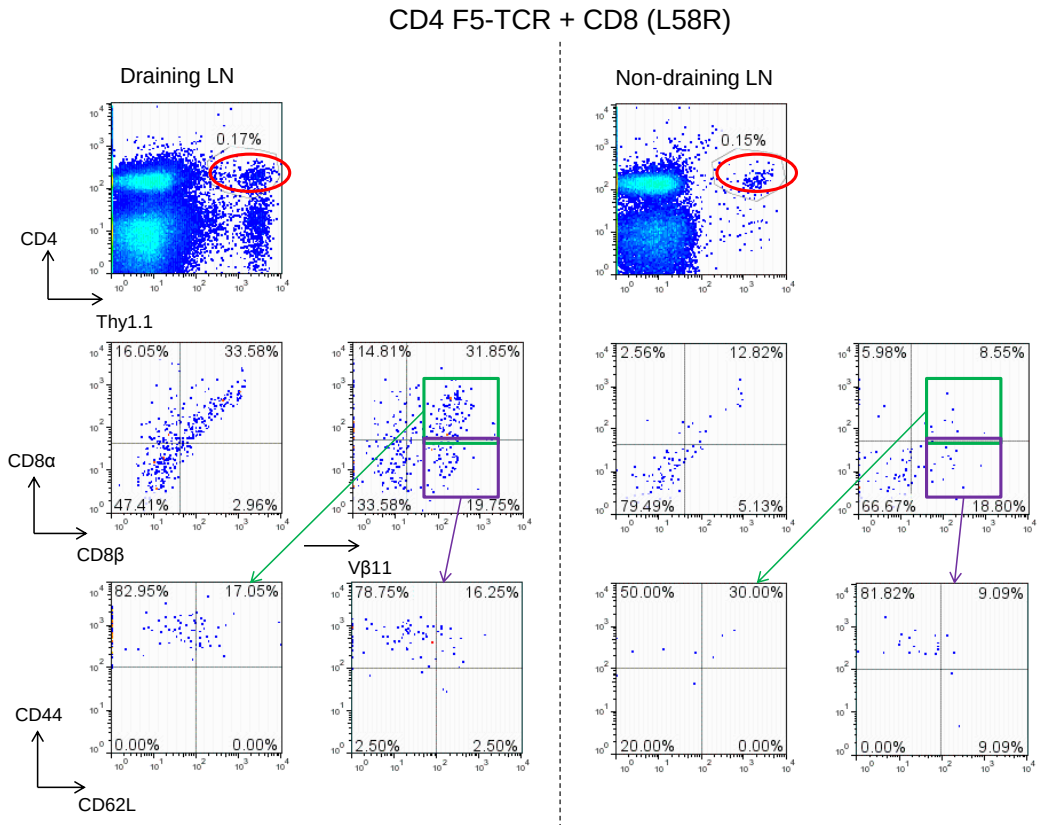


Figure 6-24 Ex vivo phenotypic analysis of lymphoid cells taken from draining (TDLN) with non-draining (NDLN) lymph nodes after Ag re-challenge in mice previously treated with thy1.1+ CD4+ CD8 L58R F5-TCR transduced T cells.
Example FACS plots shown from one surviving animal. Endogenous thy1.1neg CD4+ T cells were used as controls for gating purposes. The F5-TCR was detected by anti-Vβ11 antibodies. The cells shown were first gated on live lymphocytes and then thy1.1+ CD4+ T cells (red circle). CD8α+ (green square) and CD8α- (purple square) Vβ11+ T cells were examined separately for memory phenotype using CD44 and CD62L.

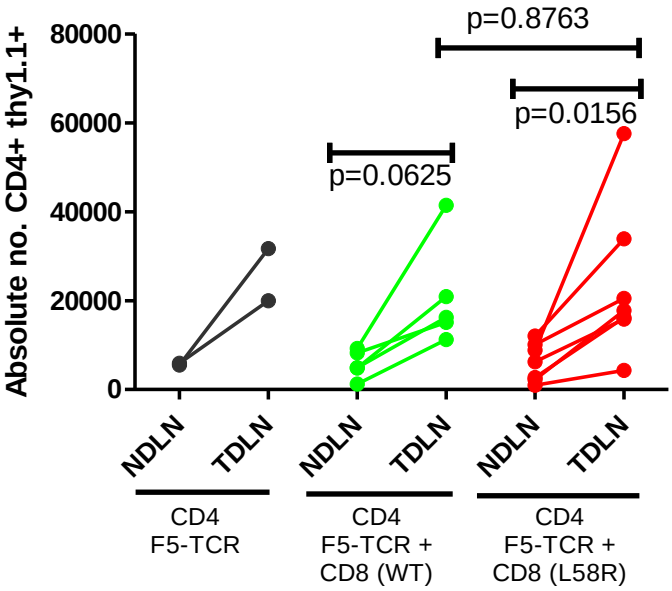


Figure 6-25 In vivo expansion of transferred thy1.1+ CD4+ T cells in tumour draining (TDLN) compared to non-draining (NDLN).
Surviving mice were re-challenged with irradiated 1 x 10^6 EL4-NPluc tumour cells injected into right lower leg and then culled for analysis 5 days later. The Thy1.1+ CD4+ T cells counts taken from the NDLN and TDLN of each individual mouse are linked.

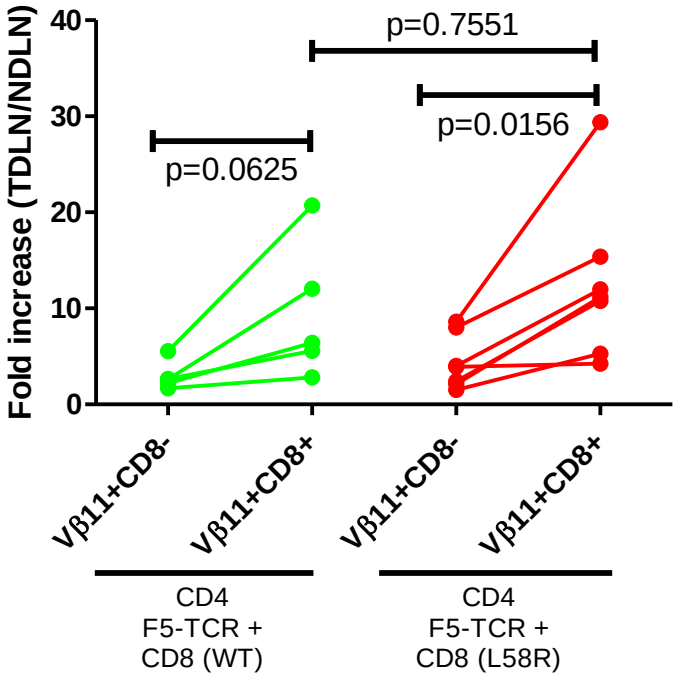


Figure 6-26 Preferential in vivo expansion of CD8+ CD4+ T cells after tumour re-challenge.
The fold increase was calculated by dividing the absolute count of the CD8- or CD8+ thy1.1+ Vβ11+ CD4+ population in TDLN divided by the absolute count of the same population in NDLN. Lines connect the values found in each individual mouse.

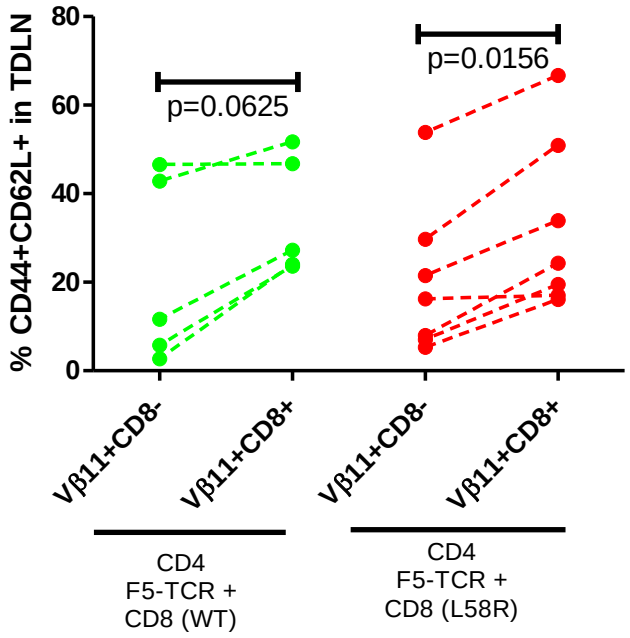


Figure 6-27 In vivo Ag re-challenge affected the proportion of central memory phenotype CD8+ CD4+ T cells isolated from tumour draining lymph nodes (TDLN).
Values were derived from FACS analysis as shown in Figures 6-23 and 6-24. Lines connect the values found from each individual mouse before and after Ag re-challenge.

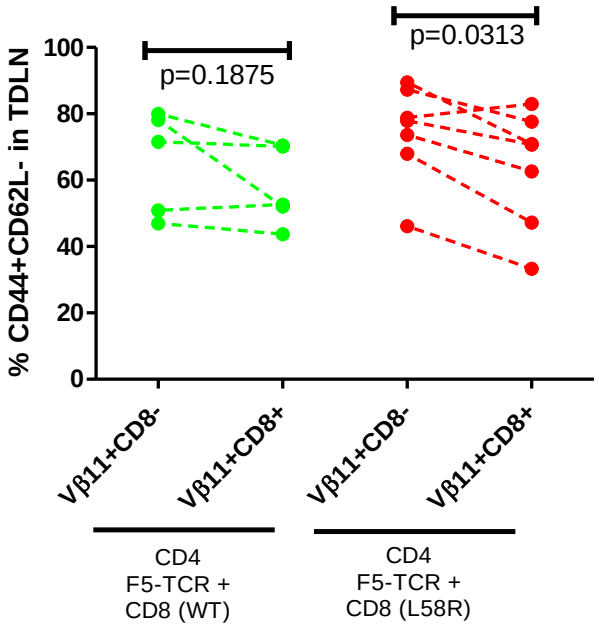


Figure 6-28 In vivo Ag re-challenge affected the proportion of effector memory phenotype CD8+ CD4+ T cells in the tumour draining lymph nodes (TDLN).
Values were derived from FACS analysis as shown in Figures 6-23 and 6-24. Lines connect the values found from each individual mouse before and after Ag re-challenge.

6.3.5 Summary

The data presented in this chapter has demonstrated the efficacy of transduced CD4+ T cells to target tumour in-vivo. Our previous study showed that F5-TCR-td CD4+ T cells without transduced CD8+ T cells could not eradicate tumour and transducing CD8α with F5-TCR into CD4+ T cells was actually less effective than using F5-TCR alone in tumour eradication, possibly through a mechanism of impaired proliferation. However, when the CD8(WT) co-receptor containing the CD8β chain was co-transduced with F5-TCR into CD4+ T cells the EL4NP tumour could be completely eradicated in mice receiving higher doses of CD4+ T cells. After tumour eradication, the transferred CD4+ T cells persisted for at least four months but CD8 co-receptor down-regulation in these cells was observed.

Two subsequent independent experiments confirmed that the CD8 co-receptor was able to augment the anti-tumour effects of CD4+ T cells transduced with the MHCI restricted F5-TCR. The CD4+ T cells transduced with the CD8 L58R co-receptor cleared tumour faster and conferred improved survival rates compared to the CD4+ T cells transduced with the CD8 WT co-receptor. Although there was significant down regulation of CD8 co-receptor in co-transduced CD4+ T-cells, the secondary proliferative responses of CD4+ T cells retaining CD8 expression were better than those that downregulated CD8 co-receptor. The CD8+ CD4+ T cells also expressed more central memory phenotype after tumour re-challenge. There was however no detectable qualitative differences between CD8 WT and the CD8 L58R co-receptor transduced CD4+ T cells in the secondary responses.

Chapter 7

Chapter 7. Discussion

Since the discovery and recognition of immunosurvelliance, various means of harnessing the immune system to target cancer have been attempted. However these early attempts to boost the immune system through vaccination such as using tumour cells with adjuvants were not effective. One important breakthrough came from the use of tumour infiltrating lymphocytes (TIL), which were expanded in-vitro following isolation from tumour biopsies and reinfused. However, this form of adoptive immunotherapy is limited by the complexity of ex vivo expansion on a per-patient basis as discussed in Section 1.6.1.

Gene transfer of TCR or CAR presents an opportunity to redirect large numbers of polyclonal T cells to target cancer cells with greater ease. TCR gene transfer relies on the isolation of TCR genes from clones of high avidity T cells, which respond to cancer in an Ag-specific manner.

The TCR genes isolated are amenable to genetic modifications such as codon optimization, introduction of cysteine residues and the replacement of constant regions with murine sequences; all of which have been shown to improve the level of TCR expression and promote correct pairing of the introduced TCR. These modifications are important as the level of TCR expression influences T cell signalling thresholds and may enhance effector function (Hart et al. 2008; van Loenen et al. 2011). The cloned TCRs used in this project incorporated many of the above molecular modifications.

Attempts to improve the 'strength' of TCR artificially by affinity maturation have generated TCR that have in excess of 100x increased affinity compared to the unmodified TCR. However, in our hands, the affinity matured TCRs when transduced into T cells did not result in improved functional avitidy (Thomas et al. 2011). It has been proposed that there is a natural ceiling for increasing TCR affinity as natural affinity exist within a narrow range (Slifka & Whitton 2001) in order to allow for serial triggering when antigen is limited (Valitutti et al. 1995).

One group modified TCR not by manipulating the antigen-binding residues but removing N-glycosylation sites within the TCR constant domain. The rationale for this is based on a study in which CD8+ T cells from MGAT5 enzyme deficient mice with reduced N-glycosylation have lower threshold for activation (Demetriou et al. 2001). To remove N-glycosylation sites, asparagine residues on the TCR constant domains were replaced with glutamine by site directed mutagenesis (Kuball et al. 2009a). Three TCR with different specificities were modified in this manner and when transduced into CD8+ T cells all showed enhanced multimer binding, increased functional avidity and improved recognition of tumour cells. Because the non-variable

region of the TCR is modified, this approach could potentially be used for TCR of any specificity.

Apart from manipulating TCR structure, the signalling threshold of transduced TCR may be improved by changes in the amount or quality of proximal signalling molecules. Examples in the literature include the up-regulation of Lck, a natural phenomenon associated with increases in functional avidity and maturation of naïve T cells after antigen encounter (Slifka & Whitton 2001). Others have modified LAT molecules to be resistant to ubiquitination resulting in improvement of T cell signalling (Balagopalan et al. 2011).

Our group tested whether the amount of endogenous CD3 available within the transduced T cell was a critical rate limiting step for assembly and expression of the introduced TCR. We performed a series of experiments where CD3 molecules were transduced together with TCR into CD8+ T cells. We demonstrated enhanced TCR expression, increased in vitro Ag-specific cytokine production and improved tumour protection in-vivo (Ahmadi et al. 2011). Other molecules involved in proximal signalling were therefore investigated, such as the CD8 co-receptor. Unlike the CD3 molecule, which is required for the expression both MHCI and MHCII restricted TCR, the CD8 co-receptor is only crucial for the function of the MHCI restricted TCR.

To date, most TCR targeting cancer antigens have been isolated from CD8+ T cells. It was hypothesized that the co-transfer of additional CD8 co-receptor (especially those with 'augmenting' mutations) together with the MHCI restricted TCR may enhance Ag-specific T cell function further. Many studies have generated specific mutations in the CD8 co-receptor to dissect its structure and function. Most of the 50+ different mutations examined resulted in inferior CD8 co-receptors (Wang et al. 2009). This may suggest that the wild-type CD8 co-receptor has been optimized during evolution and further improvements are not possible. However some anti-CD8 antibodies have improved the CD8 co-receptor effect on TCR activation possibly by optimizing the stability of the CD8 molecule for engagement with MHCI as elaborated in Section 1.7. Proof of principle that enhancing the binding affinity between CD8 and MHCI could enhance primary T cell responses has been demonstrated by Sewell et al (Wooldridge et al. 2007). The three CD8 mutations known to increase multimer staining or TCR activation in cell lines are CD8α K73A, CD8β S53L and CD8β L58R, the latter two were tested in this project.

Functional assays of transduced T cells performed in this project focused on in vitro measures of T cell avidity and responses against tumour cells expressing the cognate antigen. TCR avidity is frequently defined as the responsiveness of T cells to target cell expressing cognate antigen (Kuball et al. 2009b; Slifka & Whitton 2001). Both IFN-γ and IL-2 production were measured as both Th1 cytokines are known to be

important for anti-tumour effects. Proliferation assays were performed but gave inconsistent results with the CD8 co-receptor transduced T cells due to high background impairing the relevant comparisons. This may have been because the T cells had been strongly activated during transduction and low level proliferation persisted for several days after activation cues were removed.

Of the eight CD8β mutants tested in this project, four were MHC-binding site mutants previously described in the literature (S53L, S54V, L58R and L58R/I25A) and four were glycosylation site mutants, not previously described (T120A, T121A, T124A and TglyM). The two mutants found to be augmenting when tested in CD8β -/- T cells both contained the MHC-binding site L58R mutation. Expressing the CD8β chain containing the L58R mutation increased the maximum magnitude of IFN-γ Ag-specific responses and peptide sensitivity. These findings were consistent with the increase in Ag-specific IL-2 production from transduced BW cells as demonstrated by Devine et al. The crystal structure of murine CD8αβ in complex with H2-Db has shown that the L58 residue, previously assumed to be the contact residue for MHCI, was found instead to be a contact residue for CD8α S108. Thus the L58R substitution was thought to enhance the stability of the CD8 co-receptor heterodimer (Wang et al. 2009). When tested in the same assays the CD8β containing the S53L mutation was not found to augment the Ag-specific function of CD8β-/- T cells, which may have been related to its lower expression. The exact reason for the lower CD8β S53L expression is not known. The S54V mutated CD8β although found to have increased MHC binding affinity, had not been shown to increase Ag-specific responses in this or any previous study. Therefore increasing the MHC binding of the CD8 co-receptor may not always translate to the augment Ag-specific responses. It is possible that the MHC binding mutants may also affect the ability of the CD8 co-receptor to associate with the TCR giving rise to this discrepancy.

Removal of one of three CD8β glycosylation sites did not have an impact on the level of CD8β expression in transduced CD8β-/- T cells. However, the triple glycosylation site mutant (CD8β TglyM) when transduced into CD8β-/- T cells resulted in reduced IFN-γ T cells. This may suggest that removal of more than one O-glycan is required to affect the structural stability of the CD8 molecule. There is evidence that the CD8β stalk where these O-glycans are located interacts with the TCR connecting peptide motif (CPM) but whether the O-glycans directly interact with the CPM motif is unknown. The aim with the CD8β O-glycans was to prevent desialylation from occurring as the CD8β chain in the desialylated state is associated with improved TCR signal transduction (Section 1.4). This hypothesis was not upheld with our experimental data, which may have been because CD8+ or CD4+ T cells when activated during the transduction process had already undergone CD8β desialylation.

It is possible that the augmenting effects of the CD8β glycosylation site mutants may only be seen in naïve T cells where the CD8β chain is typically sialylated. There is some evidence that less differentiated T cells may have better anti-tumour efficacy and persist better after transfer (Gattinoni et al. 2005; Hinrichs et al. 2009). Various strategies could be used to generate less differentiated naïve T cells, for example through the use of lentiviral vectors for gene transfer (Zhou et al. 2003; Perro et al. 2010), through the manipulation of cellular pathways such as the Wnt-β catenin pathyway with the GSK kinase inhibitor (TWS119), or by using haemopoietic stem cells (HSC) for transduction (Gattinoni et al. 2009; Gattinoni et al. 2011). The CD8β glycosylation site mutants may be more useful in this setting.

The CD8β L58R augmenting mutant when transduced into CD8+ T cells did not enhance Ag-specific responses. Therefore the augmenting CD8β L58R mutant did not confer any gain of function or dominant positive effect on T cells endogenously expressing the CD8 co-receptor. It would be theoretically possible to silence production of the endogenous protein by siRNA prior to introduction of the mutated co-receptor. This approach was used to study LAT mutants conferring resistance to ubiquitylation in Jurkat cell lines and primary CD8+ T cells (Balagopalan et al. 2011). The LAT mutants were transfected into CD8+ T cells treated with siRNA, which had reduced endogenous LAT by 30%. LAT mutant transduced CD8+ T cells upregulated the activation marker CD69 more rapidly than control CD8+ T cells.

For the transduction of both F5-TCR and CD8β into CD8+ T cells a larger quad-cistronic vector (~10kb in size) containing both TCR and CD8 genes was used as described in Section 3.4. Transduction of murine T cells with the large F5-TCR-CD8αβ vector resulted in lower TCR expression and the transduced T cells demonstrated reduced Ag-specific production of IL-2 (Section 3.4.5). However, human T cells transduced with the CMV-TCR-CD8αβ vector showed good expression of molecules transcribed by all the transgenes and were of high avidity (Section 5.4). The reason for this discrepancy is unclear but had also been noted by other researchers using retroviral vectors (Dr Zamoyska personal communication) and lentiviral vectors (Kerkar et al. 2011). Studies on the resistance of murine T cells to HIV infection have identified post-entry blocks due to blocking of the pre-integration complex (Baumann et al. 2004; Tsurutani et al. 2007). It is thought that a human specific factor is necessary for integration of HIV genes into murine cells (Tsurutani et al. 2007). Although this is unlikely to be related to the failure of the F5-TCR-CD8 vector, both studies highlight that cellular differences between murine and human cell can influence differential integration of retroviruses.

One of the safety concerns for TCR gene transfer is the potential for off-target adverse effects, manifest as autoimmunity, due to mispairing of the introduced TCR

chains with endogenous TCR chains and formation of novel TCR heterodimers with unknown specificities (Thomas et al. 2011). This has been shown in mice given transduced T cells, which resulted in damage to the haemopoietic compartment, pancreatitis and colitis which was similar to graft-versus-host disease (Bendle et al. 2010). Strategies preventing TCR mispairing include TCR chain modifications, the transduction of oligoclonal T cells and ex vivo Ag-specific stimulation of transduced T cells before transfer. Although TCR-transduced CD4+ T cells may also cause off-target toxicity, none of the mice examined in this project were affected. This may be in part due to the TCR modifications previously described (Section 3.2), the lower numbers of CD4+ T cells used and the lack of exogenous IL-2 administration. Once tumour was eradicated, the surviving mice gained weight and were healthy. Co-transfer of the CD8 co-receptor enhanced tumour eradication by transduced CD4+ T cells but did not seem to cause any associated graft-versus-host like disease.

Not all adverse effects of T cell responses are caused by off-target toxicity. When normal tissues express the same antigens as the intended target, on-target toxicity may occur. This is especially relevant in the setting of cancer where tumour associated antigens are usually expressed at lower levels in some normal tissues. Examples of on-target toxicity have occurred for both TCR and CAR transduced T cells (Section 1.6). In addition, the CD8 co-receptor has recently been shown to increase the cross-reactivity of class I restricted TCR (Wooldridge et al. 2010). This study found that ILA1 CD8+ CTL were able to respond to a larger repertoire of peptides when APC contain HLA-A2 mutants with increased CD8 binding affinity. It might therefore be predicted that the CD8 co-receptor containing the 'augmenting' CD8β L58R mutation could increase TCR cross reactivity and potentially exaggerate either on- or off-target toxicities. Although no particular toxicity was seen in the mice given CD4+ T cells transduced with CD8 L58R, this could be because the level of augmentation was insufficient to induce on- or off- target toxicity or because CD8 co-receptor was down-regulated within two weeks of transfer. Nevertheless, it will be interesting to study the magnitude by which the L58R mutation enhances CD8/MHCI binding affinity and whether it causes any cross-reactivity.

It is widely acknowledged that CD8+ T cells and CD4+ T cells have distinct functional differences as described in Section 1.5. Whether transducing CD8 co-receptor would alter the biology of CD4+ T cells is an intriguing question but is thought to be unlikely as the genetic programming of peripheral CD4+ T cells is likely to be fixed. Formally addressing this question is complicated as the CD8 transduced CD4+ T cells would have to be in a resting state after activation during the transduction process which is likely to influence the gene profile. As murine T cells were difficult to be kept in culture for prolonged periods, this would mean that the transduced CD4+ T cells

would have to be transferred back into synergic mice to 'rest' for a period of time and then re-isolated for gene profiling by microarray. The small amounts of transferred CD4+ T cells persisting in the mice ($<1x10^5$ cells) after three months was unlikely to be sufficient for gene profiling. A less rigorous way of determining similarity or differences between CD4+ T cells and CD8+ T cells would be by the repertoire of cytokines or chemokines produced. However this was not performed in this project and only IFN-γ and IL-2 cytokines were measured. In murine transduced CD8+ T cells the ag-specific response resulted in small amounts of IL-2 but large amounts of IFN-γ. For murine transduced CD4+ T cells the reverse pattern is seen with large amounts of IL-2 but small amounts of IFN-γ. The low amount of IFN-γ produced is related to the small number of IFN-γ producing CD4+ T cells. This is consistent with the Ag-specific response of CD4+ T cells to Listeria and viral infections (Homann et al. 2001; Cauley et al. 2002). When CD8 is transduced into CD4+ T cells, the Ag-specific cytokine production is heightened but the pattern of high IL-2 and low IFN-γ remains suggesting that there is no significant change in biology. There was Ag-specific upregulation of CD40L, a characteristic of CD4+ T cells, with or without the CD8 co-receptor transduced in.

In human CD4+ T cells transduced with MHCI restricted TCR recognising HLA-A2/pp65, the Ag-specific response resulted in significant production of both IL-2 and IFN-α which is not different to transduced CD8+ T cells. These results were part of an extensive study of cytokine production by CD4+ and CD8+ T cells using Luminex multiplex technology and was performed by Dr S Xue in our laboratory which showed that TCR-td CD4+ T cells produce high levels of TNF-α, IL-4 and IL-5 compared to TCR transduced CD8+ T cells which produced very little of these cytokines. Co-transducing the CD8 co-receptor into CD4+ T cells only resulted in enhancement of these cytokines and did not affect the overall pattern of cytokine production by CD4+ T cells. It had been observed by another group that CD4+ T cell transduced with MHCI restricted TCR isolated from a high avidity T cell clone are multifunctional in the repertoire of cytokines produced. This was similar to the study performed our laboratory and showed production of IL-2, TNF-α, IFN-γ and CD107a (Ray et al. 2010). When the MHCII restricted TCR was used instead to transduce CD8+ T cells, it did not induce CD8+ T cells to produce large amounts of cytokines even when the CD4 co-receptor is co-transduced (van der Veken et al. 2005). Although co-transfer of CD4 co-receptor augmented the cytolytic effect of MHCII restricted TCR transduced CD8+ T cells. Therefore the transduction CD8 co-receptor into CD4+ T cells is unlikely to change the biology of the CD4+ T cells in both the murine and human systems.

The in vivo tumour protection effects of transferring CD4+ T cells were dramatic. Mice given tumour and mock transduced T cells deteriorated rapidly because of poor local control of tumour with ulceration and signs of distress indicating systemic tumour effects. Mice given tumour and TCR transduced CD4+ T cells remained healthy for prolonged periods but had to be culled because of poor local tumour control. Mice rescued with CD8 and TCR co-transduced CD4+ T cells had the best outcome in terms of both tumour regression and overall survival. Overall there was good correlation between in-vitro function and in-vivo tumour protection effects of transduced CD4+ T cells supporting the rationale of determining function first using in vitro tests before progressing of in-vivo experiments.

The mechanism(s) by which transduced CD4+ T cells afford tumour protection in this project has not yet been delineated. The CD4+ T cells were injected into the tail vein and have to migrate to the tumour site. Previous experiments looking at migration of transduced T cells using bioluminescence showed that aggregation first occurs in the lungs before appearing at the tumour site from day 5 onwards (Ahmadi et al. 2011). The aggregation of cells at the tumour site can be difficult to study as cell numbers have to be above a threshold of 1×10^4 cells/cm^2 before a reasonable signal is detected in shaved C57Bl/6 mice. High avidity CD8+ T cells were shown to have a greater propensity to aggregate at tumour sites compared to low avidity CD8+ T cells likely to contribute to greater anti-tumour efficacy (Ahmadi et al. 2011). The inference from this would be that F5-TCR and CD8 co-transduced CD4+ T cells may be superior to F5-TCR alone transduced CD4+ T cells in this aspect, but this phenomenon has not been studied in this project.

In the tumour in vivo experiments described in Chapter 6, the transduced cell populations were depleted of CD8+ T cells before transduction. This reduces the number of CD8+ T cells to less than 0.5% of the total live lymphocytes pre-transfer and makes it seem unlikely that the tumour protection was provided by the rare CD8+ T cells given the rapid rate of the tumour growth. In the condition with F5-TCR transduced CD4+ T cells, no thy1.1 CD8+ T cells were detected in the peripheral blood or lymphoid organs at various time points. In the condition with CD8 co-transduced, a population of thy1.1+ CD8+ T cells were seen that were probably related to the significant proportion of CD4neg and CD8neg cells that were incidentally transduced with CD8 co-receptor and the F5-TCR. It is unknown if these cells affect the tumour but as they exist at low levels at all the time points their role is probably limited. Despite the rigorous methodology to exclude non CD4+ T cells in this project, the existence of small numbers of anti-tumour CD8+ T cells that have escaped detection cannot be excluded. In a previously published study, minor HLA

specific donor CD8+ T cells had been found in patients transplanted with allogeneic BMT after the infusion of CD8 depleted DLI (Zorn et al. 2002).

Both transgenic and transduced CD4+ T cells as discussed above and in Section 1.5.2, can be 'multi-functional' with the ability to produce IFN-γ, TNF-α and IL-2 cytokines plus cytotoxic effects through perforin, granzyme B and degranulation (Ray et al. 2010; Quezada et al. 2010; Xie et al. 2010). Therefore it is likely that the ability of transduced CD4+ T cells to eradicate tumour is multifactorial with some factors such as production of IFN-γ and IL-2 playing a larger role (Cohen et al. 2000) and CD40-CD40L interaction playing a lesser role (Kline et al. 2012). A recent study showed that MHCII restricted TRP1-TCR transduced CD4+ T cells were more effective than MHCI restricted Pmel1-TCR transduced CD8+ T cells to eradicate large melanoma tumours (Kerkar et al. 2011). This was surprising as it had generally been accepted that CD8+ T cells play a central role in tumour eradication with the CD4+T cells playing a more supportive role (Nishimura et al. 1999). The improved tumour protection seen with CD4+ T cells compared to CD8+ T cells was likely to be due to qualitative differences rather as increasing the amount of CD8+ T cells resulted in the same findings. The anti-tumour effects of CD4+ and CD8+ T cells may be difficult to compare directly because two different TCR were used. Two studies had used the same CD8 independent TCR for transduction into CD4+ or CD8+ T cells and compared their relative impact on tumour eradication. In the first study, both CD4+ and CD8+ T cells transduced with anti-tyrosinase TCR gave similar levels of tumour protection to mice with melanoma (Frankel et al. 2010). In the second study, CD4+ and CD8+ T cells transduced with 2C-TCR also gave similar levels of tumour protection against melanoma, however CD4+ T cells transduced with affinity matured 2C-TCR (m33-TCR) had the most effective anti-tumour responses in-vivo (Soto et al. 2012). Surprisingly the CD8+ T cells transduced with the affinity matured 2C-TCR (m33-TCR) gave poor tumour protection as underwent apoptosis and was deleted in the periphery within hours unlike CD8+ T cells transduced with 2C-TCR (Engels et al. 2012). When CD4+ T cells were co-transduced with CD8 co-receptor and m33-TCR, these cells were similarly deleted in the periphery. However CD4+ T cells transduced just with m33-TCR was still detected 80 days after transfer.

The potentiating effects of CD4+ T cells on cytotoxic CD8+ T cells is well documented (Nishimura et al. 1999; Morris et al. 2005; Kessels et al. 2006) and therefore it is likely that the combination of the two T cell populations is required to effect the most effective anti-tumour effects although the relative proportion of each cell type to induce the most optimum effect is unknown. The most practical way of transducing both the CD4+ and CD8+ T cells would be to use a TCR taken from a high avidity clone and transduce bulk populations containing populations. In this setting, it is

unknown whether the MHCI restricted TCR has any advantage over the MHCII restricted TCR and may be related to the role of each population. The CD8 and TCR co-transduced CD4+ T cells may synergise with CD8+ T cells to eradicate tumour better than TCR alone transduced CD4+ T cells and this area may be subjected to further studies in the future. However, as described above, co-transfer of CD8 co-receptor may have a negative effect when used with a high affinity MHCI-restricted TCR.

In this project the transferred CD4+ T cells persisted indefinitely and were found 120 days after transfer even though small amounts ($1x10^5$ cells) were introduced in the beginning. This was similar to a study in which in vitro expanded CD4+ TIL transferred into synergic mice were detected at low frequencies (<1% of total lymphocytes) without the presence of antigen at day 90 (Wang & Plautz 2010). However a large proportion of CD8 transduced CD4+ T cells had down-regulated the CD8 co-receptor from as early as 14 days after transfer with a large proportion previously CD8+ CD4+ T cells no longer expressing detectable CD8 molecules above background. Although down-regulation of transduced molecules such as TCR and CD3 were observed previously, the down-regulation has not to such a great extent as seen with the CD8 co-receptor (Ahmadi et al. 2011). Down-regulation of CD8 molecules were also observed during in vitro experiments when around 50% of CD8 transduced CD4+ T cells no longer expressing CD8 molecules after one week of restimulation. Two possibilities could account for this; firstly the CD8 vector was in a less efficient pMX vector and CD8α and CD8β transgenes were separated by IRES. Secondly the down-regulation of CD8 could also be due to intrinsic genetic programming of the CD4+ T cells which suppresses endogenous CD8 expression and may also affect transduced CD8 expression. Nevertheless the CD4+ T cells that retain the CD8 molecule was found to have a greater ability to expand after rechallenge suggesting that the greater avidity also resulted in improved proliferative capacity.

Some studies suggest that memory T cells are superior to naïve T cells at targeting tumour for adoptive immunotherapy (Gattinoni et al. 2005; Berger et al. 2008). These may be related to the classical characteristic of memory T cells of rapid effector cytokine responses follow activation from a previous encountered antigen that is greater than that of the primary response (Chandok et al. 2007; Mohrs et al. 2005). In the situation when the tumour clearance is slow because of rapid growth, memory T cells that result from the early responses may have a role in the eventual control of tumour growth. While memory responses have been extensively studied in the infection setting, much less is known in the tumour setting. Moreover, it is also acknowledged that memory CD4+ T cells are not as well understood as the memory

CD8+ T cells (McKinstry et al. 2010; MacLeod et al. 2009). This may be related to smaller numbers of memory CD4+ T cells and their greater functional heterogeneity impeding analysis. Studies showed that both murine and human memory CD4+ T cells decrease in numbers over time unlike CD8+ memory T cells (Homann et al. 2001; Seder & Ahmed 2003; Jelley-Gibbs et al. 2008). Memory CD4+ T cells have the ability to enhance CD8+ T cell responses in both infection and tumour setting and can control re-infection through IFN-γ (North & Jung 2004; Brown et al. 2004). In Chapter 6, the CD44 and CD69 markers associated with memory T cells were analysed in the transferred thy1.1+ CD4+ T cells. As a result of non-Ag specific activation necessary for the transduction process, the pre-transferred CD4+ T cells already have CD44 expression which is maintained in-vivo after the cells were introduced into tumour bearing mice expressing cognate antigen. A recent study showed that the CD44 molecule is expressed after influenza virus exposure only in Th1 but not in Th2 or Th17 CD4+ T cells (Baaten et al. 2012). In a viral infection model, Th1 CD4+ T cells that go on to become memory T cells can be defined by the transcription pattern of high PSGL1 and low Lyt6C and Tbet but not reliably by IL7R expression (Marshall et al. 2011). The CD44 is an adhesion molecule enhances the survival of CD4+ T cells by augmenting TCR signalling when antigen levels are low (Föger et al. 2000) and by inhibiting apoptotic pathways (Mielgo et al. 2006; Baaten et al. 2010) which may explain its association with memory T cells. High levels of CD44 in transferred CD4+ T cells several months after transfer without the presence of cognate antigen was also seen by Plautz et al (Wang & Plautz 2010) which suggest that once CD44 is up-regulated, expression is permanent and present even in progeny cells.

Unlike the CD44 molecule, the transduced thy1.1 CD4+ T cells had variable expression of CD62L expression before and after transfer. Persisting transferred CD4+ T cells were mostly CD62Lneg (>85%) in the spleen and bone marrow with an enrichment of CD62L+ CD4+ T cells of 25-35% in the lymph nodes. This was similar to the study with Plautz et al when in-vitro expanded CD4+ T cells were transferred into synergic mice. CD4+ T cells that up-regulate CD62L expression after activation and are known as central memory T cells. CD62L is also known as L-selectin, an adhesion molecule involved in homing of lymphocytes into lymph nodes after activation. These central memory T cells were shown to arise as a result of asynchronous exposure to antigen from late arriving Ag-specific CD4+ T cells that are exposed to lower quantities of antigen (Catron et al. 2006). Down-regulation of CD62L on T cells can occur after activation of central memory T cells and induce trafficking away lymph nodes into peripheral sites. Central memory T cells are known to produce small amounts of effector cytokines but large amounts of IL-2 and associated with high proliferative potential. Effector memory T cells, in comparison,

produce larger amounts of effector cytokines rapidly on re-exposure to antigen. There are conflicting studies on the role of central and effector CD4+ T cells on infection and tumour immunity (Seder et al. 2008). CD62Lneg tumour specific T cells taken from tumour draining lymph nodes in mice were more effective against tumour challenge than CD62L+ T cells when transferred into synergic mice challenged with tumour (Peng et al. 2002; Wang et al. 2007). However transduced CD62L+ CD8+ T cells were more effective than transduced CD62Lneg CD8+ T cells in affording tumour in two different studies (Gattinoni et al. 2005; Berger et al. 2008). The relevance of these different cell populations are also difficult to dissect as CD62Lneg effector memory T cells can re-express CD62L and CD62L+ central memory T cells can give rise to CD62Lneg effector memory T cells. In this project, the transferred CD4+ T cells still expressing CD8 co-receptor proliferated and contained higher proportion of the central memory phenotype in tumour draining lymph nodes. The significance of the increased proportion of CD4+ T cells expressing CD62L is unknown and further experiments may be required to determine the functional qualities of CD62L+ versus CD62Lneg CD4+ T cells.

This study has shown that CD8 co-receptor can augment the functional avidity of a MHCI restricted TCR (F5-TCR) when co-transduced into CD4+ T cells. This augmenting effect is further enhanced by introducing the L58R mutation in the CD8 co-receptor. CD4+ T cells co-transduced with F5-TCR and CD8 co-receptor gave improved tumour protection and were able to eradicate tumour cells completely. These co-transduced CD4+ T cells persisted indefinitely, express the effector memory phenotype and respond to re-challenge. The information obtained from this project will have implications in the use of other MHCI restricted TCR to transduce CD4+ T cells. Firstly, it may avoid the need for the TCR to undergo affinity maturation if affinity is at the lower end of the physiological range and thus allow a greater number of MHCI restricted TCRs to be used to transduce the more dynamic CD4+ T cells for cancer immunotherapy. Secondly, it reinforces the current data that it is possible to use CD8 depleted CD4+ T cells to target tumour.

References

Aguirre-Ghiso, J.A., 2007. Models, mechanisms and clinical evidence for cancer dormancy. *Nature reviews. Cancer*, 7(11), pp.834–46.

Ahmadi, M. et al., 2011. CD3 limits the efficacy of TCR gene therapy in vivo. *Blood*, 118(13), pp.3528–3537.

Ahmadi, M. et al., 2011. CD3 limits the efficacy of TCR gene therapy in vivo. *Blood*, 118(13), pp.3528–37.

Aivazian, D. & Stern, L.J., 2000. Phosphorylation of T cell receptor zeta is regulated by a lipid dependent folding transition. *Nature structural biology*, 7(11), pp.1023–6.

Alam, S.M. et al., 1996. T-cell-receptor affinity and thymocyte positive selection. *Nature*, 381(6583), pp.616–20.

Amir, A.L. et al., 2011. PRAME-specific Allo-HLA-restricted T cells with potent antitumor reactivity useful for therapeutic T-cell receptor gene transfer. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 17(17), pp.5615–25.

Andersen, R.S. et al., 2012. Dissection of T-cell antigen specificity in human melanoma. *Cancer research*, 72(7), pp.1642–50.

Angelov, G.S., Guillaume, P. & Luescher, I.F., 2009. CD8beta knockout mice mount normal anti-viral CD8+ T cell responses--but why? *International immunology*, 21(2), pp.123–35.

Anikeeva, N. et al., 2006. Quantum dot/peptide-MHC biosensors reveal strong CD8-dependent cooperation between self and viral antigens that augment the T cell response. *Proceedings of the National Academy of Sciences of the United States of America*, 103(45), pp.16846–51.

Apte, S.H. et al., 2008. Interferon-gamma and interleukin-4 reciprocally regulate CD8 expression in CD8+ T cells. *Proceedings of the National Academy of Sciences of the United States of America*, 105(45), pp.17475–80.

Arcaro, a et al., 2001. CD8beta endows CD8 with efficient coreceptor function by coupling T cell receptor/CD3 to raft-associated CD8/p56(lck) complexes. *The Journal of experimental medicine*, 194(10), pp.1485–95.

Arnett, K.L., Harrison, S.C. & Wiley, D.C., 2004. Crystal structure of a human CD3-epsilon/delta dimer in complex with a UCHT1 single-chain antibody fragment. *Proceedings of the National Academy of Sciences of the United States of America*, 101(46), pp.16268–73.

Artyomov, M.N. et al., 2010. CD4 and CD8 binding to MHC molecules primarily acts to enhance Lck delivery. *Proceedings of the National Academy of Sciences of the United States of America*, 107(39), pp.16916–21.

Baaten, B.J.G. et al., 2010. CD44 regulates survival and memory development in Th1 cells. *Immunity*, 32(1), pp.104–15.

Baaten, B.J.G. et al., 2012. Regulation of Antigen-Experienced T Cells: Lessons from the Quintessential Memory Marker CD44. *Frontiers in immunology*, 3(February), p.23.

Bäckström, B.T. et al., 1996. A motif within the T cell receptor alpha chain constant region connecting peptide domain controls antigen responsiveness. *Immunity*, 5(5), pp.437–47.

Bäckström, B.T. et al., 1998. Positive selection through a motif in the alphabeta T cell receptor. *Science (New York, N.Y.)*, 281(5378), pp.835–8.

Baitsch, L. et al., 2011. Exhaustion of tumor-specific $CD8^+$ T cells in metastases from melanoma patients. *The Journal of clinical investigation*, 121(6), pp.2350–60.

Balagopalan, L. et al., 2011. Enhanced T-cell signaling in cells bearing linker for activation of T-cell (LAT) molecules resistant to ubiquitylation. *Proceedings of the National Academy of Sciences of the United States of America*, 108(7), pp.2885–90.

Banchereau, J. & Steinman, R.M., 1998. Dendritic cells and the control of immunity. *Nature*, 392(6673), pp.245–52.

Baum, L.G., 2002. Developing a taste for sweets. *Immunity*, 16(1), pp.5–8.

Baumann, J.G. et al., 2004. Murine T cells potently restrict human immunodeficiency virus infection. *Journal of virology*, 78(22), pp.12537–47.

Beal, A.M. et al., 2009. Kinetics of early T cell receptor signaling regulate the pathway of lytic granule delivery to the secretory domain. *Immunity*, 31(4), pp.632–42.

Beal, A.M. et al., 2008. Protein kinase C theta regulates stability of the peripheral adhesion ring junction and contributes to the sensitivity of target cell lysis by CTL. *Journal of immunology (Baltimore, Md. : 1950)*, 181(7), pp.4815–24.

Bendle, G.M. et al., 2007. A Study of T Cell Tolerance to the Tumor-Associated Antigen MDM2 : Cytokines Can Restore Antigen Responsiveness , but Not High Avidity T Cell Function. , (4).

Bendle, G.M. et al., 2010. Lethal graft-versus-host disease in mouse models of T cell receptor gene therapy. *Nature medicine*, 16(5), pp.565–70, 1p following 570.

Van den Berg, H.A. et al., 2007. Coreceptor CD8-driven modulation of T cell antigen receptor specificity. *Journal of theoretical biology*, 249(2), pp.395–408.

Berger, C. et al., 2008. Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates. *The Journal of clinical investigation*, 118(1), pp.294–305.

Berner, V. et al., 2007. IFN-gamma mediates CD4+ T-cell loss and impairs secondary antitumor responses after successful initial immunotherapy. *Nature medicine*, 13(3), pp.354–60.

Bevan, M.J., 2004. Helping the CD8(+) T-cell response. *Nature reviews. Immunology*, 4(8), pp.595–602.

Beyers, A.D., Spruyt, L.L. & Williams, A.F., 1992. Molecular associations between the T-lymphocyte antigen receptor complex and the surface antigens CD2, CD4, or CD8 and CD5. *Proceedings of the National Academy of Sciences of the United States of America*, 89(7), pp.2945–9.

Blankenstein, T. et al., 2012. The determinants of tumour immunogenicity. *Nature reviews. Cancer*, 12(4), pp.307–13.

Bos, R. & Sherman, L.A., 2010. CD4 + T-Cell Help in the Tumor Milieu Is Required for Recruitment and Cytolytic Function of CD8 + T Lymphocytes Recruitment and Cytolytic Function of CD8 + T Lymphocytes. , pp.8368–8377.

Bosselut, R. et al., 2000. Role of CD8beta domains in CD8 coreceptor function: importance for MHC I binding, signaling, and positive selection of CD8+ T cells in the thymus. *Immunity*, 12(4), pp.409–18.

Bosselut, R. et al., 2003. Unraveling a revealing paradox: Why major histocompatibility complex I-signaled thymocytes “paradoxically” appear as CD4+8lo transitional cells during positive selection of CD8+ T cells. *The Journal of experimental medicine*, 197(12), pp.1709–19.

Boulter, J.M. et al., 2003. Stable, soluble T-cell receptor molecules for crystallization and therapeutics. *Protein Engineering Design and Selection*, 16(9), pp.707–711.

Brown, D.M., Román, E. & Swain, S.L., 2004. CD4 T cell responses to influenza infection. *Seminars in immunology*, 16(3), pp.171–7.

Brugnera, E. et al., 2000. Coreceptor reversal in the thymus: signaled CD4+8+ thymocytes initially terminate CD8 transcription even when differentiating into CD8+ T cells. *Immunity*, 13(1), pp.59–71.

Bunnell, S.C. et al., 2002. T cell receptor ligation induces the formation of dynamically regulated signaling assemblies. *The Journal of cell biology*, 158(7), pp.1263–75.

Buslepp, J., Wang, H., et al., 2003. A correlation between TCR Valpha docking on MHC and CD8 dependence: implications for T cell selection. *Immunity*, 19(4), pp.595–606.

Buslepp, J., Kerry, S.E., et al., 2003. High affinity xenoreactive TCR:MHC interaction recruits CD8 in absence of binding to MHC. *Journal of immunology (Baltimore, Md. : 1950)*, 170(1), pp.373–83.

Call, M.E. et al., 2002. The organizing principle in the formation of the T cell receptor-CD3 complex. *Cell*, 111(7), pp.967–79.

Campanelli, R. et al., 2002. Human CD8 co-receptor is strictly involved in MHC-peptide tetramer-TCR binding and T cell activation. *International immunology*, 14(1), pp.39–44.

Casabó, L.G. et al., 1994. T cell activation results in physical modification of the mouse CD8 beta chain. *Journal of immunology (Baltimore, Md. : 1950)*, 152(2), pp.397–404.

Castellino, F. & Germain, R.N., 2006. Cooperation between CD4+ and CD8+ T cells: when, where, and how. *Annual review of immunology*, 24, pp.519–40.

Catron, D.M. et al., 2006. CD4+ T cells that enter the draining lymph nodes after antigen injection participate in the primary response and become central-memory cells. *The Journal of experimental medicine*, 203(4), pp.1045–54.

Cauley, L.S. et al., 2002. Cutting edge: virus-specific CD4+ memory T cells in nonlymphoid tissues express a highly activated phenotype. *Journal of immunology (Baltimore, Md. : 1950)*, 169(12), pp.6655–8.

Cawthon, A.G. & Alexander-Miller, M.A., 2002. Optimal colocalization of TCR and CD8 as a novel mechanism for the control of functional avidity. *Journal of immunology (Baltimore, Md. : 1950)*, 169(7), pp.3492–8.

Cebecauer, M. et al., 2005. CD8+ cytotoxic T lymphocyte activation by soluble major histocompatibility complex-peptide dimers. *The Journal of biological chemistry*, 280(25), pp.23820–8.

Chandok, M.R. et al., 2007. A biochemical signature for rapid recall of memory CD4 T cells. *Journal of immunology (Baltimore, Md. : 1950)*, 179(6), pp.3689–98.

Chang, H.-C., Tan, K. & Hsu, Y.-M., 2006. CD8alphabeta has two distinct binding modes of interaction with peptide-major histocompatibility complex class I. *The Journal of biological chemistry*, 281(38), pp.28090–6.

Chaplin, D.D., 2010. Overview of the immune response. *The Journal of allergy and clinical immunology*, 125(2 Suppl 2), pp.S3–23.

Chhabra, A. et al., 2008. CD4+CD25- T Cells Transduced to Express MHC Class I-Restricted Epitope-Specific TCR Synthesize Th1 Cytokines and Exhibit MHC Class I-Restricted Cytolytic Effector Function in a Human Melanoma Model. *J. Immunol.*, 181(2), pp.1063–1070.

Chua, I., Quinti, I. & Grimbacher, B., 2008. Lymphoma in common variable immunodeficiency: interplay between immune dysregulation, infection and genetics. *Current opinion in hematology*, 15(4), pp.368–74.

Cohen, C.J. et al., 2006. Enhanced antitumor activity of murine-human hybrid T-cell receptor (TCR) in human lymphocytes is associated with improved pairing and TCR/CD3 stability. *Cancer research*, 66(17), pp.8878–86.

Cohen, C.J. et al., 2007. Enhanced antitumor activity of T cells engineered to express T-cell receptors with a second disulfide bond. *Cancer research*, 67(8), pp.3898–903.

Cohen, P.A. et al., 2000. CD4+ T cells in adoptive immunotherapy and the indirect mechanism of tumor rejection. *Critical reviews in immunology*, 20(1), pp.17–56.

Collins, A. et al., 2011. RUNX transcription factor-mediated association of Cd4 and Cd8 enables coordinate gene regulation. *Immunity*, 34(3), pp.303–14.

Collins, R.H. et al., 1997. Donor leukocyte infusions in 140 patients with relapsed malignancy after allogeneic bone marrow transplantation. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, 15(2), pp.433–44.

Connolly, J.M. et al., 1990. Recognition by CD8 on cytotoxic T lymphocytes is ablated by several substitutions in the class I alpha 3 domain: CD8 and the T-cell receptor recognize the same class I molecule. *Proceedings of the National Academy of Sciences of the United States of America*, 87(6), pp.2137–41.

Corthay, A. et al., 2005. Primary antitumor immune response mediated by CD4+ T cells. *Immunity*, 22(3), pp.371–83.

Corti, D. & Lanzavecchia, A., 2013. Broadly Neutralizing Antiviral Antibodies. *Annual review of immunology*, 31, pp.705–42.

Crooks, M.E. & Littman, D.R., 1994. Disruption of T lymphocyte positive and negative selection in mice lacking the CD8 beta chain. *Immunity*, 1(4), pp.277–85.

Curiel, T.J. et al., 2004. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nature medicine*, 10(9), pp.942–9.

Daniels, M. a et al., 2001. CD8 binding to MHC class I molecules is influenced by T cell maturation and glycosylation. *Immunity*, 15(6), pp.1051–61.

Daniels, M.A. & Jameson, S.C., 2000. Critical role for CD8 in T cell receptor binding and activation by peptide/major histocompatibility complex multimers. *The Journal of experimental medicine*, 191(2), pp.335–46.

Demetriou, M. et al., 2001. Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation. *Nature*, 409(6821), pp.733–9.

Demotte, N. et al., 2010. A galectin-3 ligand corrects the impaired function of human CD4 and CD8 tumor-infiltrating lymphocytes and favors tumor rejection in mice. *Cancer research*, 70(19), pp.7476–88.

Devine, L. et al., 2004. Location of the epitope for an anti-CD8alpha antibody 53.6.7 which enhances CD8alpha-MHC class I interaction indicates antibody stabilization of a higher affinity CD8 conformation. *Immunology letters*, 93(2-3), pp.123–30.

Devine, L. et al., 2006. Mapping the binding site on CD8 beta for MHC class I reveals mutants with enhanced binding. *Journal of immunology (Baltimore, Md. : 1950)*, 177(6), pp.3930–8.

Devine, L. et al., 1999. Orientation of the Ig domains of CD8 alpha beta relative to MHC class I. *Journal of immunology (Baltimore, Md. : 1950)*, 162(2), pp.846–51.

Dighe, A.S. et al., 1994. Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN gamma receptors. *Immunity*, 1(6), pp.447–56.

Donnelly, M.L. et al., 2001. Analysis of the aphthovirus 2A/2B polyprotein “cleavage” mechanism indicates not a proteolytic reaction, but a novel translational effect: a putative ribosomal “skip”. *The Journal of general virology*, 82(Pt 5), pp.1013–25.

Doucey, M.-A. et al., 2003. CD3 delta establishes a functional link between the T cell receptor and CD8. *The Journal of biological chemistry*, 278(5), pp.3257–64.

Dudley, M.E. et al., 2005. Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, 23(10), pp.2346–57.

Dudley, M.E. et al., Adoptive transfer of cloned melanoma-reactive T lymphocytes for the treatment of patients with metastatic melanoma. *Journal of immunotherapy (Hagerstown, Md. : 1997)*, 24(4), pp.363–73.

Dunn, G.P. et al., 2002. Cancer immunoediting: from immunosurveillance to tumor escape. *Nature immunology*, 3(11), pp.991–8.

Ebert, P.J.R., Ehrlich, L.I.R. & Davis, M.M., 2008. Low ligand requirement for deletion and lack of synapses in positive selection enforce the gauntlet of thymic T cell maturation. *Immunity*, 29(5), pp.734–45.

Edwards, L.J. et al., 2012. Insights intoT cell recognition of antigen : significance of two-dimensional kinetic parameters. , 3(April), pp.1–9.

Ehrlich, P., 1909. About the current state of carcinoma research. *Ned Tijdscher Geneeskd*.

Engels, B. et al., 2012. Long-term persistence of CD4(+) but rapid disappearance of CD8(+) T cells expressing an MHC class I-restricted TCR of nanomolar affinity. *Molecular therapy : the journal of the American Society of Gene Therapy*, 20(3), pp.652–60.

Engels, B. et al., 2003. Retroviral Vectors for High-Level Transgene Expression in T Lymphocytes. , 1168, pp.1155–1168.

Erard, F. et al., 1993. Switch of CD8 T cells to noncytolytic CD8-CD4- cells that make TH2 cytokines and help B cells. *Science (New York, N.Y.)*, 260(5115), pp.1802–5.

Erman, B. et al., 2006. Coreceptor signal strength regulates positive selection but does not determine CD4/CD8 lineage choice in a physiologic in vivo model. *Journal of immunology (Baltimore, Md. : 1950)*, 177(10), pp.6613–25.

Faroudi, M. et al., 2003. Lytic versus stimulatory synapse in cytotoxic T lymphocyte/target cell interaction: manifestation of a dual activation threshold. *Proceedings of the National Academy of Sciences of the United States of America*, 100(24), pp.14145–50.

Fearon, E.R. et al., 1990. Interleukin-2 production by tumor cells bypasses T helper function in the generation of an antitumor response. *Cell*, 60(3), pp.397–403.

Feau, S. et al., 2011. Autocrine IL-2 is required for secondary population expansion of CD8 + memory T cells. , 12(9).

Fehling, H.J. et al., 1995. Crucial role of the pre-T-cell receptor alpha gene in development of alpha beta but not gamma delta T cells. *Nature*, 375(6534), pp.795–8.

Feinerman, O. et al., 2008. Variability and robustness in T cell activation from regulated heterogeneity in protein levels. *Science (New York, N.Y.)*, 321(5892), pp.1081–4.

Föger, N., Marhaba, R. & Zöller, M., 2000. CD44 supports T cell proliferation and apoptosis by apposition of protein kinases. *European journal of immunology*, 30(10), pp.2888–99.

Fragoso, R. et al., 2003. Lipid raft distribution of CD4 depends on its palmitoylation and association with Lck, and evidence for CD4-induced lipid raft aggregation as an additional mechanism to enhance CD3 signaling. *Journal of immunology (Baltimore, Md. : 1950)*, 170(2), pp.913–21.

Frankel, T.L. et al., 2010. Both CD4 and CD8 T cells mediate equally effective in vivo tumor treatment when engineered with a highly avid TCR targeting tyrosinase. *Journal of immunology (Baltimore, Md. : 1950)*, 184(11), pp.5988–98.

Frey, A.B., 1995. Rat mammary adenocarcinoma 13762 expressing IFN-gamma elicits antitumor CD4+ MHC class II-restricted T cells that are cytolytic in vitro and

tumoricidal in vivo. *Journal of immunology (Baltimore, Md. : 1950)*, 154(9), pp.4613–22.

Fung-Leung, W.P. et al., 1994. Reduced thymic maturation but normal effector function of CD8+ T cells in CD8 beta gene-targeted mice. *The Journal of experimental medicine*, 180(3), pp.959–67.

Furler, S. et al., 2001. Recombinant AAV vectors containing the foot and mouth disease virus 2A sequence confer efficient bicistronic gene expression in cultured cells and rat substantia nigra neurons. *Gene therapy*, 8(11), pp.864–73.

Gabrilovich, D.I. & Nagaraj, S., 2009. Myeloid-derived suppressor cells as regulators of the immune system. *Nature reviews. Immunology*, 9(3), pp.162–74.

Gagnon, E. et al., 2010. Response multilayered control of T cell receptor phosphorylation. *Cell*, 142(5), pp.669–71.

Gakamsky, D.M. et al., 2005. CD8 kinetically promotes ligand binding to the T-cell antigen receptor. *Biophysical journal*, 89(3), pp.2121–33.

Gakamsky, D.M., Luescher, I.F. & Pecht, I., 2004. T cell receptor-ligand interactions: a conformational preequilibrium or an induced fit. *Proceedings of the National Academy of Sciences of the United States of America*, 101(24), pp.9063–6.

Gallagher, P.F., Fazekas de St Groth, B. & Miller, J.F., 1989. CD4 and CD8 molecules can physically associate with the same T-cell receptor. *Proceedings of the National Academy of Sciences of the United States of America*, 86(24), pp.10044–8.

Gangadharan, D. & Cheroutre, H., 2004. The CD8 isoform CD8alphaalpha is not a functional homologue of the TCR co-receptor CD8alphabeta. *Current opinion in immunology*, 16(3), pp.264–70.

Gao, G.F. et al., 1997. Crystal structure of the complex between human CD8alpha(alpha) and HLA-A2. *Nature*, 387(6633), pp.630–4.

Gao, L. et al., 2000. Selective elimination of leukemic CD34(+) progenitor cells by cytotoxic T lymphocytes specific for WT1. *Blood*, 95(7), pp.2198–203.

Garboczi, D.N. et al., 1996. Assembly, specific binding, and crystallization of a human TCR-alphabeta with an antigenic Tax peptide from human T lymphotropic virus type 1 and the class I MHC molecule HLA-A2. *Journal of immunology (Baltimore, Md. : 1950)*, 157(12), pp.5403–10.

Garcia, K.C., Degano, M., et al., 1996. An alphabeta T cell receptor structure at 2.5 A and its orientation in the TCR-MHC complex. *Science (New York, N.Y.)*, 274(5285), pp.209–19.

Garcia, K.C., Scott, C.A., et al., 1996. CD8 enhances formation of stable T-cell receptor/MHC class I molecule complexes. *Nature*, 384(6609), pp.577–81.

Garrido, F., Cabrera, T. & Aptsiauri, N., 2010. “Hard” and “soft” lesions underlying the HLA class I alterations in cancer cells: implications for immunotherapy. *International journal of cancer. Journal international du cancer*, 127(2), pp.249–56.

Gascoigne, N.R.J., 2002. T-cell differentiation: MHC class I’s sweet tooth lost on maturity. *Current biology : CB*, 12(3), pp.R99–R101.

Gattinoni, L. et al., 2011. A human memory T cell subset with stem cell-like properties. *Nature medicine*, 17(10), pp.1290–1297.

Gattinoni, L. et al., 2005. Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumor efficacy of adoptively transferred CD8+ T cells. *The Journal of clinical investigation*, 115(6), pp.1616–26.

Gattinoni, L. et al., 2009. Wnt signaling arrests effector T cell differentiation and generates CD8+ memory stem cells. *Nature medicine*, 15(7), pp.808–13.

Getnet, D. et al., 2009. Tumor recognition and self-recognition induce distinct transcriptional profiles in antigen-specific CD4 T cells. *Journal of immunology (Baltimore, Md. : 1950)*, 182(8), pp.4675–85.

Gibbings, D. & Befus, A.D., 2009. CD4 and CD8: an inside-out coreceptor model for innate immune cells. *Journal of leukocyte biology*, 86(2), pp.251–9.

Gil, D. et al., 2002. Recruitment of Nck by CD3 epsilon reveals a ligand-induced conformational change essential for T cell receptor signaling and synapse formation. *Cell*, 109(7), pp.901–12.

Gil, D. et al., 2005. T cell receptor engagement by peptide-MHC ligands induces a conformational change in the CD3 complex of thymocytes. *The Journal of experimental medicine*, 201(4), pp.517–22.

De Goër de Herve, M.-G. et al., 2010. Direct CD4 help provision following interaction of memory CD4 and CD8 T cells with distinct antigen-presenting dendritic cells. *Journal of immunology (Baltimore, Md. : 1950)*, 185(2), pp.1028–36.

Greenberg, P.D., Cheever, M.A. & Fefer, A., 1981. Eradication of disseminated murine leukemia by chemoimmunotherapy with cyclophosphamide and adoptively transferred immune syngeneic Lyt-1+2- lymphocytes. *The Journal of experimental medicine*, 154(3), pp.952–63.

Greenberg, P.D., Kern, D.E. & Cheever, M.A., 1985. Therapy of disseminated murine leukemia with cyclophosphamide and immune Lyt-1+,2- T cells. Tumor eradication does not require participation of cytotoxic T cells. *The Journal of experimental medicine*, 161(5), pp.1122–34.

Grisotto, M.G. et al., 2001. Most parasite-specific CD8+ cells in Trypanosoma cruzi-infected chronic mice are down-regulated for T-cell receptor-alphabeta and CD8 molecules. *Immunology*, 102(2), pp.209–17.

Hart, D.P. et al., 2008. Retroviral transfer of a dominant TCR prevents surface expression of a large proportion of the endogenous TCR repertoire in human T cells. *Gene therapy*, 15(8), pp.625–31.

Hayakawa, Y. et al., 2011. Early activation and interferon-γ production of tumor-infiltrating mature CD27 high natural killer cells. *Cancer science*, 102(11), pp.1967–71.

He, Xiao et al., 2005. The zinc finger transcription factor Th-POK regulates CD4 versus CD8 T-cell lineage commitment. *Nature*, 433(7028), pp.826–33.

Henrickson, S.E. et al., 2008. T cell sensing of antigen dose governs interactive behavior with dendritic cells and sets a threshold for T cell activation. *Nature immunology*, 9(3), pp.282–91.

Hermans, I.F. et al., 1998. Antigen expressed on tumor cells fails to elicit an immune response, even in the presence of increased numbers of tumor-specific cytotoxic T lymphocyte precursors. *Cancer research*, 58(17), pp.3909–17.

Hervas-Stubbs, S. et al., 2007. TLR3 ligand stimulates fully functional memory CD8+ T cells in the absence of CD4+ T-cell help. *Blood*, 109(12), pp.5318–26.

Heslop, H.E. et al., 2010. Long-term outcome of EBV-specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients. *Blood*, 115(5), pp.925–35.

Hinrichs, C.S. et al., 2009. Adoptively transferred effector cells derived from naive rather than central memory CD8+ T cells mediate superior antitumor immunity. *Proceedings of the National Academy of Sciences of the United States of America*, 106(41), pp.17469–74.

Hock, H. et al., 1991. Interleukin 7 induces CD4+ T cell-dependent tumor rejection. *The Journal of experimental medicine*, 174(6), pp.1291–8.

Hogquist, K.A. et al., 1994. T cell receptor antagonist peptides induce positive selection. *Cell*, 76(1), pp.17–27.

Holler, P.D. & Kranz, D.M., 2003. Quantitative Analysis of the Contribution of TCR / pepMHC Affinity and CD8 to T Cell Activation. , 18, pp.255–264.

Homann, D., Teyton, L. & Oldstone, M.B., 2001. Differential regulation of antiviral T-cell immunity results in stable CD8+ but declining CD4+ T-cell memory. *Nature medicine*, 7(8), pp.913–9.

Huang, Y. et al., 2011. Mucosal memory $CD8^+$ T cells are selected in the periphery by an MHC class I molecule. *Nature immunology*, 12(11), pp.1086–95.

Hunder, N.N. et al., 2008. Treatment of metastatic melanoma with autologous CD4+ T cells against NY-ESO-1. *The New England journal of medicine*, 358(25), pp.2698–703.

Hung, K. et al., 1998. The central role of CD4(+) T cells in the antitumor immune response. *The Journal of experimental medicine*, 188(12), pp.2357–68.

Irvine, D.J. et al., 2002. Direct observation of ligand recognition by T cells. *Nature*, 419(6909), pp.845–9.

Isaaz, S. et al., 1995. Serial killing by cytotoxic T lymphocytes: T cell receptor triggers degranulation, re-filling of the lytic granules and secretion of lytic proteins via a non-granule pathway. *European journal of immunology*, 25(4), pp.1071–9.

Jelley-Gibbs, D.M. et al., Influencing the fates of CD4 T cells on the path to memory: lessons from influenza. *Immunology and cell biology*, 86(4), pp.343–52.

Jiang, N. et al., 2011. Two-stage cooperative T cell receptor-peptide major histocompatibility complex-CD8 trimolecular interactions amplify antigen discrimination. *Immunity*, 34(1), pp.13–23.

Julias, J.G., Hash, D. & Pathak, V.K., 1995. E- vectors: development of novel self-inactivating and self-activating retroviral vectors for safer gene therapy. *Journal of virology*, 69(11), pp.6839–46.

Kägi, D. et al., 1994. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature*, 369(6475), pp.31–7.

Kambayashi, T. et al., 2001. IL-2 down-regulates the expression of TCR and TCR-associated surface molecules on CD8(+) T cells. *European journal of immunology*, 31(11), pp.3248–54.

Kao, C., Daniels, M.A. & Jameson, S.C., 2005. Loss of CD8 and TCR binding to Class I MHC ligands following T cell activation. *International immunology*, 17(12), pp.1607–17.

Kaplan, D.H. et al., 1998. Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. *Proceedings of the National Academy of Sciences of the United States of America*, 95(13), pp.7556–61.

Kast, W.M. et al., 1989. Eradication of adenovirus E1-induced tumors by E1A-specific cytotoxic T lymphocytes. *Cell*, 59(4), pp.603–14.

Kerkar, S.P. et al., 2011. Genetic engineering of murine CD8+ and CD4+ T cells for preclinical adoptive immunotherapy studies. *Journal of immunotherapy (Hagerstown, Md. : 1997)*, 34(4), pp.343–52.

Kern, P. et al., 1999. Expression, purification, and functional analysis of murine ectodomain fragments of CD8alphaalpha and CD8alphabeta dimers. *The Journal of biological chemistry*, 274(38), pp.27237–43.

Kern, P.S. et al., 1998. Structural basis of CD8 coreceptor function revealed by crystallographic analysis of a murine CD8alphaalpha ectodomain fragment in complex with H-2Kb. *Immunity*, 9(4), pp.519–30.

Kershaw, M.H. et al., 2006. A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 12(20 Pt 1), pp.6106–15.

Kessels, H.W.H.G. et al., 2006. Generation of T cell help through a MHC class I-restricted TCR. *Journal of immunology (Baltimore, Md. : 1950)*, 177(2), pp.976–82.

Kieback, E. et al., 2008. A safeguard eliminates T cell receptor gene-modified autoreactive T cells after adoptive transfer. *Proceedings of the National Academy of Sciences of the United States of America*, 105(2), pp.623–8.

Kieback, E. & Uckert, W., 2010. Enhanced T cell receptor gene therapy for cancer. *Expert opinion on biological therapy*, 10(5), pp.749–62.

Kienzle, N. et al., 2002. A clonal culture system demonstrates that IL-4 induces a subpopulation of noncytolytic T cells with low CD8, perforin, and granzyme expression. *Journal of immunology (Baltimore, Md. : 1950)*, 168(4), pp.1672–81.

Kim, S.T. et al., 2010. Distinctive CD3 heterodimeric ectodomain topologies maximize antigen-triggered activation of alpha beta T cell receptors. *Journal of immunology (Baltimore, Md. : 1950)*, 185(5), pp.2951–9.

Kim, T.W. et al., 2007. Oral administration of high molecular mass poly-gamma-glutamate induces NK cell-mediated antitumor immunity. *Journal of immunology (Baltimore, Md. : 1950)*, 179(2), pp.775–80.

Kisielow, P. et al., 1988. Positive selection of antigen-specific T cells in thymus by restricting MHC molecules. *Nature*, 335(6192), pp.730–3.

Kizaki, T. et al., 1991. Immune suppression induced by protoscoleces of Echinococcus multilocularis in mice. Evidence for the presence of CD8dull suppressor cells in spleens of mice intraperitoneally infected with E. multilocularis. *Journal of immunology (Baltimore, Md. : 1950)*, 147(5), pp.1659–66.

Kjer-Nielsen, L. et al., 2004. Crystal structure of the human T cell receptor CD3 epsilon gamma heterodimer complexed to the therapeutic mAb OKT3. *Proceedings of the National Academy of Sciences of the United States of America*, 101(20), pp.7675–80.

Kline, J. et al., 2012. Cellular and molecular requirements for rejection of B16 melanoma in the setting of regulatory T cell depletion and homeostatic proliferation. *Journal of immunology (Baltimore, Md. : 1950)*, 188(6), pp.2630–42.

Knutson, K.L., Schiffman, K. & Disis, M.L., 2001. Immunization with a HER-2/neu helper peptide vaccine generates HER-2/neu CD8 T-cell immunity in cancer patients. *The Journal of clinical investigation*, 107(4), pp.477–84.

Kochenderfer, J.N. et al., 2011. B-cell depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical trial of anti-CD19 chimeric-antigen-receptor-transduced T cells. *Blood*, pp.blood–2011–10–384388–.

Koebel, C.M. et al., 2007. Adaptive immunity maintains occult cancer in an equilibrium state. *Nature*, 450(7171), pp.903–7.

Krammer, P.H., Arnold, R. & Lavrik, I.N., 2007. Life and death in peripheral T cells. *Nature reviews. Immunology*, 7(7), pp.532–42.

Krogsgaard, M. et al., 2005. Agonist/endogenous peptide-MHC heterodimers drive T cell activation and sensitivity. *Nature*, 434(7030), pp.238–43.

Krogsgaard, M., Juang, J. & Davis, M.M., 2007. A role for "self" in T-cell activation. *Seminars in immunology*, 19(4), pp.236–44.

Kuball, J. et al., 2005. Cooperation of human tumor-reactive CD4+ and CD8+ T cells after redirection of their specificity by a high-affinity p53A2.1-specific TCR. *Immunity*, 22(1), pp.117–29.

Kuball, J. et al., 2007. Facilitating matched pairing and expression of TCR chains introduced into human T cells. *Blood*, 109(6), pp.2331–8.

Kuball, J. et al., 2009a. Increasing functional avidity of TCR-redirected T cells by removing defined N-glycosylation sites in the TCR constant domain. *The Journal of experimental medicine*, 206(2), pp.463–75.

Kuball, J. et al., 2009b. Increasing functional avidity of TCR-redirected T cells by removing defined N-glycosylation sites in the TCR constant domain. *The Journal of experimental medicine*, 206(2), pp.463–75.

Kuhns, M.S. et al., 2010. Evidence for a functional sidedness to the alphabetaTCR. *Proceedings of the National Academy of Sciences of the United States of America*, 107(11), pp.5094–9.

Kuhns, M.S. & Davis, M.M., 2007. Disruption of extracellular interactions impairs T cell receptor-CD3 complex stability and signaling. *Immunity*, 26(3), pp.357–69.

Kvistborg, P. et al., 2012. TIL therapy broadens the tumor-reactive CD8(+) T cell compartment in melanoma patients. *Oncoimmunology*, 1(4), pp.409–418.

Lamers, C.H.J. et al., 2006. Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, 24(13), pp.e20–2.

Laugel, B. et al., 2007. Different T Cell Receptor Affinity Thresholds and CD8 Coreceptor Dependence Govern Cytotoxic T Lymphocyte Activation and Tetramer Binding Properties *. , 282(33), pp.23799–23810.

Laugel, B. et al., 2011. The multiple roles of the CD8 coreceptor in T cell biology: opportunities for the selective modulation of self-reactive cytotoxic T cells. *Journal of leukocyte biology*, 90(6), pp.1089–99.

Lee, K.-H. et al., 2002. T cell receptor signaling precedes immunological synapse formation. *Science (New York, N.Y.)*, 295(5559), pp.1539–42.

Lee, P.U. et al., 2000. Role of 2CT cell receptor residues in the binding of self- and allo-major histocompatibility complexes. *The Journal of experimental medicine*, 191(8), pp.1355–64.

Lin, K.Y. et al., 1996. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. *Cancer research*, 56(1), pp.21–6.

Liu, X. & Bosselut, R., 2004. Duration of TCR signaling controls CD4-CD8 lineage differentiation in vivo. *Nature immunology*, 5(3), pp.280–8.

Liu, Y. et al., 2003. The crystal structure of a TL/CD8alphaalpha complex at 2.1 A resolution: implications for modulation of T cell activation and memory. *Immunity*, 18(2), pp.205–15.

Lobe, C.G. et al., 1986. Novel serine proteases encoded by two cytotoxic T lymphocyte-specific genes. *Science (New York, N.Y.)*, 232(4752), pp.858–61.

Van Loenen, M.M. et al., 2011. Optimization of the HA-1-specific T-cell receptor for gene therapy of hematologic malignancies. *Haematologica*, 96(3), pp.477–81.

Luescher, I.F. et al., 1995. CD8 modulation of T-cell antigen receptor-ligand interactions on living cytotoxic T lymphocytes. *Nature*, 373(6512), pp.353–6.

MacLeod, M.K.L. et al., 2009. CD4 memory T cells: what are they and what can they do? *Seminars in immunology*, 21(2), pp.53–61.

Madakamutil, L.T. et al., 2004. CD8alphaalpha-mediated survival and differentiation of CD8 memory T cell precursors. *Science (New York, N.Y.)*, 304(5670), pp.590–3.

Maile, R. et al., 2005. Peripheral “CD8 tuning” dynamically modulates the size and responsiveness of an antigen-specific T cell pool in vivo. *Journal of immunology (Baltimore, Md. : 1950)*, 174(2), pp.619–27.

Mallaun, M. et al., 2008. The T cell receptor’s alpha-chain connecting peptide motif promotes close approximation of the CD8 coreceptor allowing efficient signal initiation. *Journal of immunology (Baltimore, Md. : 1950)*, 180(12), pp.8211–21.

Marshall, H.D. et al., 2011. Differential expression of Ly6C and T-bet distinguish effector and memory Th1 CD4(+) cell properties during viral infection. *Immunity*, 35(4), pp.633–46.

McKinstry, K.K., Strutt, T.M. & Swain, S.L., 2010. The potential of CD4 T-cell memory. *Immunology*, 130(1), pp.1–9.

McNicol, A.-M. et al., 2007. CD8alpha/alpha homodimers fail to function as co-receptor for a CD8-dependent TCR. *European journal of immunology*, 37(6), pp.1634–41.

Van der Merwe, P.A. & Dushek, O., 2011. Mechanisms for T cell receptor triggering. *Nature reviews. Immunology*, 11(1), pp.47–55.

Mielgo, A. et al., 2006. A novel antiapoptotic mechanism based on interference of Fas signaling by CD44 variant isoforms. *Cell death and differentiation*, 13(3), pp.465–77.

Mikolajczyk, M.G. et al., 2004. Characterization of antibodies to capsular polysaccharide antigens of Haemophilus influenzae type b and Streptococcus pneumoniae in human immune globulin intravenous preparations. *Clinical and diagnostic laboratory immunology*, 11(6), pp.1158–64.

Mingueneau, M. et al., 2008. The proline-rich sequence of CD3epsilon controls T cell antigen receptor expression on and signaling potency in preselection CD4+CD8+ thymocytes. *Nature immunology*, 9(5), pp.522–32.

Mitra, A.K. et al., 2004. Supine orientation of a murine MHC class I molecule on the membrane bilayer. *Current biology : CB*, 14(8), pp.718–24.

Mohrs, K. et al., 2005. A two-step process for cytokine production revealed by IL-4 dual-reporter mice. *Immunity*, 23(4), pp.419–29.

Monach, P.A. et al., 1995. A unique tumor antigen produced by a single amino acid substitution. *Immunity*, 2(1), pp.45–59.

Moody, A.M. et al., 2001. Developmentally regulated glycosylation of the CD8alphabeta coreceptor stalk modulates ligand binding. *Cell*, 107(4), pp.501–12.

Moody, A.M. et al., 2003. Sialic acid capping of CD8beta core 1-O-glycans controls thymocyte-major histocompatibility complex class I interaction. *The Journal of biological chemistry*, 278(9), pp.7240–6.

Moore, T. V et al., 2009. Relationship between CD8-dependent antigen recognition, T cell functional avidity, and tumor cell recognition. *Cancer immunology, immunotherapy : CII*, 58(5), pp.719–28.

Moreau, H.D. et al., 2012. Dynamic In Situ Cytometry Uncovers T Cell Receptor Signaling during Immunological Synapses and Kinapses In Vivo. *Immunity*, 37(2), pp.351–63.

Morgan, R.A. et al., 2006. Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science (New York, N.Y.)*, 314(5796), pp.126–9.

Morgan, R.A. et al., 2010. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. *Molecular therapy : the journal of the American Society of Gene Therapy*, 18(4), pp.843–51.

Morris, E.C. et al., 2005. A critical role of T cell antigen receptor-transduced MHC class I-restricted helper T cells in tumor protection. *Proceedings of the National Academy of Sciences of the United States of America*, 102(22), pp.7934–9.

Morris, G.P. & Allen, P.M., 2012. How the TCR balances sensitivity and specificity for the recognition of self and pathogens. *Nature immunology*, 13(2), pp.121–8.

Mueller, S.N. et al., 2012. Memory T Cell Subsets, Migration Patterns, and Tissue Residence. *Annual review of immunology*, 31, pp.137–61.

Muranski, P. et al., 2008. Tumor-specific Th17-polarized cells eradicate large established melanoma. *Blood*, 112(2), pp.362–73.

Naeher, D. et al., 2007. A constant affinity threshold for T cell tolerance. *The Journal of experimental medicine*, 204(11), pp.2553–9.

Naeher, D., Luescher, I.F. & Palmer, E., 2002. A role for the alpha-chain connecting peptide motif in mediating TCR-CD8 cooperation. *Journal of immunology (Baltimore, Md. : 1950)*, 169(6), pp.2964–70.

Nagai, K. et al., 2011. Aurora kinase A-specific T-cell receptor gene transfer redirects T lymphocytes to display effective anti-leukemia reactivity. *Blood*, 119(2), pp.368–376.

Naviaux, R.K. et al., 1996. The pCL vector system: rapid production of helper-free, high-titer, recombinant retroviruses. *Journal of virology*, 70(8), pp.5701–5.

Nelson, B.H., 2008. The impact of T-cell immunity on ovarian cancer outcomes. *Immunological reviews*, 222, pp.101–16.

Nemazee, D., 2000. Receptor selection in B and T lymphocytes. *Annual review of immunology*, 18, pp.19–51.

Nishimura, T. et al., 1999. Distinct role of antigen-specific T helper type 1 (Th1) and Th2 cells in tumor eradication in vivo. *The Journal of experimental medicine*, 190(5), pp.617–27.

North, R.J. & Jung, Y.-J., 2004. Immunity to tuberculosis. *Annual review of immunology*, 22, pp.599–623.

Odom, L.F. et al., 1978. Remission of relapsed leukaemia during a graft-versus-host reaction. A “graft-versus-leukaemia reaction” in man? *Lancet*, 2(8089), pp.537–40.

Ogino, S. et al., 2011. Cancer immunology--analysis of host and tumor factors for personalized medicine. *Nature reviews. Clinical oncology*, 8(12), pp.711–9.

OLD, L.J. & BOYSE, E.A., 1964. IMMUNOLOGY OF EXPERIMENTAL TUMORS. *Annual review of medicine*, 15, pp.167–86.

Opitz, C.A. et al., 2011. An endogenous tumour-promoting ligand of the human aryl hydrocarbon receptor. *Nature*, 478(7368), pp.197–203.

Palmer, E. & Naeher, D., 2009. Affinity threshold for thymic selection through a T-cell receptor-co-receptor zipper. *Nature reviews. Immunology*, 9(3), pp.207–13.

Pappu, B.P. & Shrikant, P. a, 2004. Alteration of cell surface sialylation regulates antigen-induced naive CD8+ T cell responses. *Journal of immunology (Baltimore, Md. : 1950)*, 173(1), pp.275–84.

Pardoll, D.M. & Topalian, S.L., 1998. The role of CD4+ T cell responses in antitumor immunity. *Current opinion in immunology*, 10(5), pp.588–94.

Park, J. et al., 2007. “ Coreceptor tuning ”: cytokine signals transcriptionally tailor CD8 coreceptor expression to the self-specificity of the TCR. , 8(10), pp.1049–1059.

Park, J.R. et al., 2007. Adoptive transfer of chimeric antigen receptor re-directed cytolytic T lymphocyte clones in patients with neuroblastoma. *Molecular therapy : the journal of the American Society of Gene Therapy*, 15(4), pp.825–33.

Pasare, C. & Medzhitov, R., 2005. Control of B-cell responses by Toll-like receptors. *Nature*, 438(7066), pp.364–8.

Pascale, M.C. et al., 1992. Biosynthesis and oligosaccharide structure of human CD8 glycoprotein expressed in a rat epithelial cell line. *The Journal of biological chemistry*, 267(14), pp.9940–7.

Pasternack, M.S. & Eisen, H.N., A novel serine esterase expressed by cytotoxic T lymphocytes. *Nature*, 314(6013), pp.743–5.

Pear, W.S., Tu, L. & Stein, P.L., 2004. Lineage choices in the developing thymus : choosing the T and NKT pathways. , pp.167–173.

Pecht, I. & Gakamsky, D.M., 2005. Spatial coordination of CD8 and TCR molecules controls antigen recognition by CD8+ T-cells. *FEBS letters*, 579(15), pp.3336–41.

Peng, L. et al., 2002. Tumor-induced L-selectinhigh suppressor T cells mediate potent effector T cell blockade and cause failure of otherwise curative adoptive immunotherapy. *Journal of immunology (Baltimore, Md. : 1950)*, 169(9), pp.4811–21.

Perez-Diez, A. et al., 2007. CD4 cells can be more efficient at tumor rejection than CD8 cells. *Blood*, 109(12), pp.5346–54.

Perro, M. et al., 2010. Generation of multi-functional antigen-specific human T-cells by lentiviral TCR gene transfer. *Gene therapy*, 17(6), pp.721–32.

Pierce, S.K. & Liu, W., 2010. The tipping points in the initiation of B cell signalling: how small changes make big differences. *Nature reviews. Immunology*, 10(11), pp.767–77.

Pilotte, L. et al., 2012. Reversal of tumoral immune resistance by inhibition of tryptophan 2,3-dioxygenase. *Proceedings of the National Academy of Sciences of the United States of America*, 109(7), pp.2497–502.

Potter, T.A. et al., 2001. Formation of supramolecular activation clusters on fresh ex vivo CD8+ T cells after engagement of the T cell antigen receptor and CD8 by antigen-presenting cells. *Proceedings of the National Academy of Sciences of the United States of America*, 98(22), pp.12624–9.

Pradeu, T. & Carosella, E.D., 2006. On the definition of a criterion of immunogenicity. *Proceedings of the National Academy of Sciences of the United States of America*, 103(47), pp.17858–61.

Prévost-Blondel, A. et al., 2000. Differential requirement of perforin and IFN-gamma in CD8 T cell-mediated immune responses against B16.F10 melanoma cells expressing a viral antigen. *European journal of immunology*, 30(9), pp.2507–15.

Pule, M.A. et al., 2008. Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. *Nature medicine*, 14(11), pp.1264–70.

Purbhoo, M.A. et al., 2004. T cell killing does not require the formation of a stable mature immunological synapse. *Nature immunology*, 5(5), pp.524–30.

Purbhoo, M.A. et al., 2001. The Human CD8 Coreceptor Effects Cytotoxic T Cell Activation and Antigen Sensitivity Primarily by Mediating Complete Phosphorylation of the T Cell Receptor Chain *. , 276(35), pp.32786–32792.

Qin, Z. et al., 2003. A critical requirement of interferon gamma-mediated angiostasis for tumor rejection by CD8+ T cells. *Cancer research*, 63(14), pp.4095–100.

Qin, Z. & Blankenstein, T., 2000. CD4+ T cell--mediated tumor rejection involves inhibition of angiogenesis that is dependent on IFN gamma receptor expression by nonhematopoietic cells. *Immunity*, 12(6), pp.677–86.

Quezada, S.A. et al., 2010. Tumor-reactive CD4(+) T cells develop cytotoxic activity and eradicate large established melanoma after transfer into lymphopenic hosts. *The Journal of experimental medicine*, 207(3), pp.637–50.

Ray, S. et al., 2010. MHC-I-restricted melanoma antigen specific TCR-engineered human CD4+ T cells exhibit multifunctional effector and helper responses, in vitro. *Clinical immunology (Orlando, Fla.)*, 136(3), pp.338–47.

Renard, V. et al., 1996. CD8 beta increases CD8 coreceptor function and participation in TCR-ligand binding. *The Journal of experimental medicine*, 184(6), pp.2439–44.

Restifo, N.P., Dudley, M.E. & Rosenberg, S.A., 2012. Adoptive immunotherapy for cancer: harnessing the T cell response. *Nature reviews. Immunology*, 12(4), pp.269–81.

Rettig, L. et al., 2009. An essential role for the stalk region of CD8 beta in the coreceptor function of CD8. *Journal of immunology (Baltimore, Md. : 1950)*, 182(1), pp.121–9.

Riddell, S.R. et al., 1992. Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. *Science (New York, N.Y.)*, 257(5067), pp.238–41.

Riddell, S.R. & Greenberg, P.D., 1995. Principles for adoptive T cell therapy of human viral diseases. *Annual review of immunology*, 13, pp.545–86.

Robbins, P.F. et al., 2008. Single and dual amino acid substitutions in TCR CDRs can enhance antigen-specific T cell functions. *Journal of immunology (Baltimore, Md. : 1950)*, 180(9), pp.6116–31.

Robbins, P.F. et al., 2011. Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, 29(7), pp.917–24.

Rooney, C.M. et al., 1995. Use of gene-modified virus-specific T lymphocytes to control Epstein-Barr-virus-related lymphoproliferation. *Lancet*, 345(8941), pp.9–13.

Rosenberg, S.A. et al., 2011. Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 17(13), pp.4550–7.

Rosenberg, S.A. & Dudley, M.E., 2004. Cancer regression in patients with metastatic melanoma after the transfer of autologous antitumor lymphocytes. *Proceedings of the National Academy of Sciences of the United States of America*, 101 Suppl (suppl_2), pp.14639–45.

Rudd, P.M. et al., 1999. Roles for glycosylation of cell surface receptors involved in cellular immune recognition. *Journal of molecular biology*, 293(2), pp.351–66.

Rudolph, M.G., Stanfield, R.L. & Wilson, I.A., 2006. How TCRs bind MHCs, peptides, and coreceptors. *Annual review of immunology*, 24, pp.419–66.

Ruffell, B. et al., 2012. Leukocyte composition of human breast cancer. *Proceedings of the National Academy of Sciences of the United States of America*, 109(8), pp.2796–801.

Rybakin, V. et al., 2011. CD8αα and -αβ isotypes are equally recruited to the immunological synapse through their ability to bind to MHC class I. *EMBO reports*, 12(12), pp.1251–6.

Sadovnikova, E. & Stauss, H.J., 1996. Peptide-specific cytotoxic T lymphocytes restricted by nonself major histocompatibility complex class I molecules: reagents for tumor immunotherapy. *Proceedings of the National Academy of Sciences of the United States of America*, 93(23), pp.13114–8.

Sanz, I. et al., 2008. Phenotypic and functional heterogeneity of human memory B cells. *Seminars in immunology*, 20(1), pp.67–82.

Sarafova, S.D. et al., 2005. Modulation of coreceptor transcription during positive selection dictates lineage fate independently of TCR/coreceptor specificity. *Immunity*, 23(1), pp.75–87.

Savoldo, B. et al., 2011. CD28 costimulation improves expansion and persistence of chimeric antigen receptor-modified T cells in lymphoma patients. *The Journal of clinical investigation*, 121(5), pp.1822–6.

Schietinger, A. et al., 2010. Bystander killing of cancer requires the cooperation of CD4(+) and CD8(+) T cells during the effector phase. *The Journal of experimental medicine*, 207(11), pp.2469–77.

Schilham, M.W. et al., 1993. Alloreactive cytotoxic T cells can develop and function in mice lacking both CD4 and CD8. *European journal of immunology*, 23(6), pp.1299–304.

Schmitz, J.E. et al., 1998. Expression of the CD8alpha beta-heterodimer on CD8(+) T lymphocytes in peripheral blood lymphocytes of human immunodeficiency virus- and human immunodeficiency virus+ individuals. *Blood*, 92(1), pp.198–206.

Scholten, K.B.J. et al., 2006. Codon modification of T cell receptors allows enhanced functional expression in transgenic human T cells. *Clinical immunology (Orlando, Fla.)*, 119(2), pp.135–45.

Schreiber, R.D., Old, L.J. & Smyth, M.J., 2011. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science (New York, N.Y.)*, 331(6024), pp.1565–70.

Schumacher, T.N.M., 2001. Immunotherapy through TCR gene transfer. *Nature immunology*, 2(10), pp.957–961.

Sebzda, E. et al., 1999. Selection of the T cell repertoire. *Annual review of immunology*, 17, pp.829–74.

Seder, R.A. & Ahmed, R., 2003. Similarities and differences in CD4+ and CD8+ effector and memory T cell generation. *Nature immunology*, 4(9), pp.835–42.

Seder, R.A., Darrah, P.A. & Roederer, M., 2008. T-cell quality in memory and protection: implications for vaccine design. *Nature reviews. Immunology*, 8(4), pp.247–58.

Seliger, B., Ruiz-Cabello, F. & Garrido, F., 2008. IFN inducibility of major histocompatibility antigens in tumors. *Advances in cancer research*, 101, pp.249–76.

Shafer-Weaver, K. a et al., 2009. Immunity to murine prostatic tumors: continuous provision of T-cell help prevents CD8 T-cell tolerance and activates tumor-infiltrating dendritic cells. *Cancer research*, 69(15), pp.6256–64.

Shankaran, V. et al., 2001. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature*, 410(6832), pp.1107–11.

Shaw, A.S. et al., 1989. The lck tyrosine protein kinase interacts with the cytoplasmic tail of the CD4 glycoprotein through its unique amino-terminal domain. *Cell*, 59(4), pp.627–36.

Shore, D.A. et al., 2005. Glycosylation and the function of the T cell co-receptor CD8. *Advances in experimental medicine and biology*, 564, pp.71–84.

Shrikant, P., Khoruts, A. & Mescher, M.F., 1999. CTLA-4 blockade reverses CD8+ T cell tolerance to tumor by a CD4+ T cell- and IL-2-dependent mechanism. *Immunity*, 11(4), pp.483–93.

Simpson, A.A. et al., 2011. Structural and energetic evidence for highly peptide-specific tumor antigen targeting via allo-MHC restriction. *Proceedings of the National Academy of Sciences*, 108(52), pp.21176–81.

Singer, A., 2002. New perspectives on a developmental dilemma: the kinetic signaling model and the importance of signal duration for the CD4/CD8 lineage decision. *Current opinion in immunology*, 14(2), pp.207–15.

Singer, A., Adoro, S. & Park, J.-H., 2008. Lineage fate and intense debate: myths, models and mechanisms of CD4- versus CD8-lineage choice. *Nature reviews. Immunology*, 8(10), pp.788–801.

Slifka, M.K. & Whitton, J.L., 2000. Activated and memory CD8+ T cells can be distinguished by their cytokine profiles and phenotypic markers. *Journal of immunology (Baltimore, Md. : 1950)*, 164(1), pp.208–16.

Slifka, M.K. & Whitton, J.L., 2001. Functional avidity maturation of CD8(+) T cells without selection of higher affinity TCR. *Nature immunology*, 2(8), pp.711–7.

Smith-Garvin, J.E., Koretzky, G.A. & Jordan, M.S., 2009. T cell activation. *Annual review of immunology*, 27, pp.591–619.

Song, D.-G. et al., 2011. CD27 costimulation augments the survival and anti-tumor activity of redirected human T cells in vivo. *Blood*, 119(3), pp.696–706.

Soto, C.M. et al., 2012. MHC-class I-restricted CD4 T cells: a nanomolar affinity TCR has improved anti-tumor efficacy in vivo compared to the micromolar wild-type TCR. *Cancer immunology, immunotherapy : CII*.

Stanislawski, T. et al., 2001. Circumventing tolerance to a human MDM2-derived tumor antigen by TCR gene transfer. *Nature immunology*, 2(10).

Stauss, H.J. et al., 2007. Monoclonal T-cell receptors: new reagents for cancer therapy. *Molecular therapy : the journal of the American Society of Gene Therapy*, 15(10), pp.1744–50.

Staveley-O'Carroll, K. et al., 1998. Induction of antigen-specific T cell anergy: An early event in the course of tumor progression. *Proceedings of the National Academy of Sciences of the United States of America*, 95(3), pp.1178–83.

Stone, J.D. et al., 2011. Opposite effects of endogenous peptide-MHC class I on T cell activity in the presence and absence of CD8. *Journal of immunology (Baltimore, Md. : 1950)*, 186(9), pp.5193–200.

Stritesky, G.L., Jameson, S.C. & Hogquist, K.A., 2012. Selection of self-reactive T cells in the thymus. *Annual review of immunology*, 30, pp.95–114.

Sun, G. et al., 2005. The zinc finger protein cKrox directs CD4 lineage differentiation during intrathymic T cell positive selection. *Nature immunology*, 6(4), pp.373–81.

Sun, J. & Kavathas, P.B., 1997. Comparison of the roles of CD8 alpha alpha and CD8 alpha beta in interaction with MHC class I. *Journal of immunology (Baltimore, Md. : 1950)*, 159(12), pp.6077–82.

Sun, Z.J. et al., 2001. Mechanisms contributing to T cell receptor signaling and assembly revealed by the solution structure of an ectodomain fragment of the CD3 epsilon gamma heterodimer. *Cell*, 105(7), pp.913–23.

Sun, Z.-Y.J. et al., 2004. Solution structure of the CD3epsilondelta ectodomain and comparison with CD3epsilongamma as a basis for modeling T cell receptor topology and signaling. *Proceedings of the National Academy of Sciences of the United States of America*, 101(48), pp.16867–72.

Suzuki, S. et al., 1992. Biochemical evidence of the physical association of the majority of CD3 delta chains with the accessory/co-receptor molecules CD4 and CD8 on nonactivated T lymphocytes. *European journal of immunology*, 22(10), pp.2475–9.

Sykulev, Y., 2010. T cell receptor signaling kinetics takes the stage. *Science signaling*, 3(153), p.pe50.

Szymczak, A.L. et al., 2005. The CD3epsilon proline-rich sequence, and its interaction with Nck, is not required for T cell development and function. *Journal of immunology (Baltimore, Md. : 1950)*, 175(1), pp.270–5.

Tailor, P. et al., 2008. The proline-rich sequence of CD3epsilon as an amplifier of low-avidity TCR signaling. *Journal of immunology (Baltimore, Md. : 1950)*, 181(1), pp.243–55.

Takada, K. & Jameson, S.C., 2009. Self-class I MHC molecules support survival of naive CD8 T cells, but depress their functional sensitivity through regulation of CD8 expression levels. *The Journal of experimental medicine*, 206(10), pp.2253–69.

Takada, S. & Engleman, E.G., 1987. Evidence for an association between CD8 molecules and the T cell receptor complex on cytotoxic T cells. *Journal of immunology (Baltimore, Md. : 1950)*, 139(10), pp.3231–5.

Taniuchi, I. et al., 2002. Differential requirements for Runx proteins in CD4 repression and epigenetic silencing during T lymphocyte development. *Cell*, 111(5), pp.621–33.

Teague, R.M. et al., 2008. Article Peripheral CD8 + T Cell Tolerance to Self-Proteins Is Regulated Proximally at the T Cell Receptor.

Teh, H.S. et al., 1989. Deletion of autospecific T cells in T cell receptor (TCR) transgenic mice spares cells with normal TCR levels and low levels of CD8 molecules. *The Journal of experimental medicine*, 169(3), pp.795–806.

Teh, H.S. et al., 1988. Thymic major histocompatibility complex antigens and the alpha beta T-cell receptor determine the CD4/CD8 phenotype of T cells. *Nature*, 335(6187), pp.229–33.

Teng, M.W.L. et al., 2008. Immune-mediated dormancy: an equilibrium with cancer. *Journal of leukocyte biology*, 84(4), pp.988–93.

Thomas, S. et al., 2011. Human T cells expressing affinity-matured TCR display accelerated responses but fail to recognize low density of MHC-peptide antigen. *Blood*, 118(2), pp.319–29.

Thome, M. et al., 1996. The p56lck SH2 domain mediates recruitment of CD8/p56lck to the activated T cell receptor/CD3/zeta complex. *European journal of immunology*, 26(9), pp.2093–2100.

Till, B.G. et al., 2008. Adoptive immunotherapy for indolent non-Hodgkin lymphoma and mantle cell lymphoma using genetically modified autologous CD20-specific T cells. *Blood*, 112(6), pp.2261–71.

Trédan, O. et al., 2007. Drug resistance and the solid tumor microenvironment. *Journal of the National Cancer Institute*, 99(19), pp.1441–54.

Tsuji, T. et al., 2005. Generation of tumor-specific, HLA class I-restricted human Th1 and Tc1 cells by cell engineering with tumor peptide-specific T-cell receptor genes. *Blood*, 106(2), pp.470–6.

Tsurutani, N. et al., 2007. Nuclear import of the preintegration complex is blocked upon infection by human immunodeficiency virus type 1 in mouse cells. *Journal of virology*, 81(2), pp.677–88.

Turner, J.M. et al., 1990. Interaction of the unique N-terminal region of tyrosine kinase p56lck with cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs. *Cell*, 60(5), pp.755–65.

Uttenthal, B.J. et al., 2012. Challenges in T cell receptor gene therapy. *The journal of gene medicine*, 14(6), pp.386–99.

Uyttenhove, C. et al., 2003. Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. *Nature medicine*, 9(10), pp.1269–74.

Vajdic, C.M. & van Leeuwen, M.T., 2009. Cancer incidence and risk factors after solid organ transplantation. *International journal of cancer. Journal international du cancer*, 125(8), pp.1747–54.

Valitutti, S. et al., 1995. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature*, 375(6527), pp.148–51.

Varma, R. et al., 2006. T cell receptor-proximal signals are sustained in peripheral microclusters and terminated in the central supramolecular activation cluster. *Immunity*, 25(1), pp.117–27.

Van der Veken, L.T. et al., 2005. HLA class II restricted T-cell receptor gene transfer generates CD4+ T cells with helper activity as well as cytotoxic capacity. *Gene therapy*, 12(23), pp.1686–95.

Velica, P., 2012. Turning mTOR down in TCR gene therapy: the path to long-term protection? *Immunology*, 137, p.690.

Viola, A. et al., 1997. Quantitative contribution of CD4 and CD8 to T cell antigen receptor serial triggering. *The Journal of experimental medicine*, 186(10), pp.1775–9.

Walia, V. et al., 2012. Delving into somatic variation in sporadic melanoma. *Pigment cell & melanoma research*, 25(2), pp.155–70.

Wang, H. et al., 2010. Tonic ubiquitylation controls T-cell receptor:CD3 complex expression during T-cell development. *The EMBO journal*, 29(7), pp.1285–98.

Wang, L.-X. et al., 2007. Adoptive transfer of tumor-primed, in vitro-activated, CD4+ T effector cells (TEs) combined with CD8+ TEs provides intratumoral TE proliferation and synergistic antitumor response. *Blood*, 109(11), pp.4865–76.

Wang, L.-X. & Plautz, G.E., 2010. Tumor-primed, in vitro-activated CD4+ effector T cells establish long-term memory without exogenous cytokine support or ongoing antigen exposure. *Journal of immunology (Baltimore, Md. : 1950)*, 184(10), pp.5612–8.

Wang, R., Natarajan, K. & Margulies, D.H., 2009. Structural basis of the CD8 alpha beta/MHC class I interaction: focused recognition orients CD8 beta to a T cell proximal position. *Journal of immunology (Baltimore, Md. : 1950)*, 183(4), pp.2554–64.

Wang, R.F., 2001. The role of MHC class II-restricted tumor antigens and CD4+ T cells in antitumor immunity. *Trends in immunology*, 22(5), pp.269–76.

Wheeler, C.J. et al., 1998. Mechanisms of CD8beta-mediated T cell response enhancement: interaction with MHC class I/beta2-microglobulin and functional coupling to TCR/CD3. *Journal of immunology (Baltimore, Md. : 1950)*, 160(9), pp.4199–207.

Wiedemann, A. et al., 2006. Cytotoxic T lymphocytes kill multiple targets simultaneously via spatiotemporal uncoupling of lytic and stimulatory synapses. *Proceedings of the National Academy of Sciences of the United States of America*, 103(29), pp.10985–90.

Wiest, D.L. et al., 1993. Regulation of T cell receptor expression in immature CD4+CD8+ thymocytes by p56lck tyrosine kinase: basis for differential signaling by CD4 and CD8 in immature thymocytes expressing both coreceptor molecules. *The Journal of experimental medicine*, 178(5), pp.1701–12.

Wiest, D.L. et al., 1996. TCR activation of ZAP70 is impaired in CD4+CD8+ thymocytes as a consequence of intrathymic interactions that diminish available p56lck. *Immunity*, 4(5), pp.495–504.

Willemsen, R. et al., 2005. Redirecting human CD4+ T lymphocytes to the MHC class I-restricted melanoma antigen MAGE-A1 by TCR alphabeta gene transfer requires CD8alpha. *Gene therapy*, 12(2), pp.140–6.

Williams, C.B. et al., 1999. A kinetic threshold between negative and positive selection based on the longevity of the T cell receptor-ligand complex. *The Journal of experimental medicine*, 189(10), pp.1531–44.

Williams, M.A., Ravkov, E. V & Bevan, M.J., 2008. Rapid culling of the CD4+ T cell repertoire in the transition from effector to memory. *Immunity*, 28(4), pp.533–45.

Willimsky, G. et al., 2008. Immunogenicity of premalignant lesions is the primary cause of general cytotoxic T lymphocyte unresponsiveness. *The Journal of experimental medicine*, 205(7), pp.1687–700.

Willimsky, G. & Blankenstein, T., 2005. Sporadic immunogenic tumours avoid destruction by inducing T-cell tolerance. *Nature*, 437(7055), pp.141–6.

Witte, T., Spoerl, R. & Chang, H.C., 1999. The CD8beta ectodomain contributes to the augmented coreceptor function of CD8alphabeta heterodimers relative to CD8alphaalpha homodimers. *Cellular immunology*, 191(2), pp.90–6.

Wong, J.S. et al., 2003. Stalk region of beta-chain enhances the coreceptor function of CD8. *Journal of immunology (Baltimore, Md. : 1950)*, 171(2), pp.867–74.

Wooldridge, L. et al., 2003. Anti-CD8 antibodies can inhibit or enhance peptide-MHC class I (pMHCI) multimer binding: this is paralleled by their effects on CTL activation and occurs in the absence of an interaction between pMHCI and CD8 on the cell surface. *Journal of immunology (Baltimore, Md. : 1950)*, 171(12), pp.6650–60.

Wooldridge, L. et al., 2010. CD8 Controls T Cell Cross-Reactivity.

Wooldridge, L. et al., 2007. Enhanced immunogenicity of CTL antigens through mutation of the CD8 binding MHC class I invariant region. *European journal of immunology*, 37(5), pp.1323–33.

Workman, P. et al., 2010. Guidelines for the welfare and use of animals in cancer research. *British journal of cancer*, 102(11), pp.1555–77.

Wu, L.C. et al., 2002. Two-step binding mechanism for T-cell receptor recognition of peptide MHC. *Nature*, 418(6897), pp.552–6.

Wyer, J.R. et al., 1999. T cell receptor and coreceptor CD8 alphaalpha bind peptide-MHC independently and with distinct kinetics. *Immunity*, 10(2), pp.219–25.

Xiao, Z., Mescher, M.F. & Jameson, S.C., 2007. Detuning CD8 T cells : down-regulation of CD8 expression , tetramer binding , and response during CTL activation. , 204(11), pp.2667–2677.

Xie, Y. et al., 2010. Naive tumor-specific CD4(+) T cells differentiated in vivo eradicate established melanoma. *The Journal of experimental medicine*, 207(3), pp.651–67.

Xu, H. & Littman, D.R., 1993. A kinase-independent function of Lck in potentiating antigen-specific T cell activation. *Cell*, 74(4), pp.633–43.

Xue, S.-A. et al., 2010. Development of a Wilms' tumor antigen-specific T-cell receptor for clinical trials: engineered patient's T cells can eliminate autologous leukemia blasts in NOD/SCID mice. *Haematologica*, 95(1), pp.126–34.

Xue, S.-A. et al., 2005. Elimination of human leukemia cells in NOD/SCID mice by WT1-TCR gene-transduced human T cells. *Blood*, 106(9), pp.3062–7.

Xue, S.-A. et al., 2013. Human MHC Class I-restricted high avidity CD4(+) T cells generated by co-transfer of TCR and CD8 mediate efficient tumor rejection in vivo. *Oncoimmunology*, 2(1), p.e22590.

Yee, C. et al., 2000. Melanocyte destruction after antigen-specific immunotherapy of melanoma: direct evidence of t cell-mediated vitiligo. *The Journal of experimental medicine*, 192(11), pp.1637–44.

Yokosuka, T. et al., 2005. Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap70 and SLP-76. *Nature immunology*, 6(12), pp.1253–62.

Yu, Q. et al., 2003. In vitro evidence that cytokine receptor signals are required for differentiation of double positive thymocytes into functionally mature CD8+ T cells. *The Journal of experimental medicine*, 197(4), pp.475–87.

Zamoyska, R. et al., 1989. Inability of CD8 alpha' polypeptides to associate with p56lck correlates with impaired function in vitro and lack of expression in vivo. *Nature*, 342(6247), pp.278–81.

Zamoyska, R. & Parnes, J.R., 1988. A CD8 polypeptide that is lost after passing the Golgi but before reaching the cell surface: a novel sorting mechanism. *The EMBO journal*, 7(8), pp.2359–67.

Zehn, D., Lee, S.Y. & Bevan, M.J., 2009. Complete but curtailed T-cell response to very low-affinity antigen. *Nature*, 458(7235), pp.211–4.

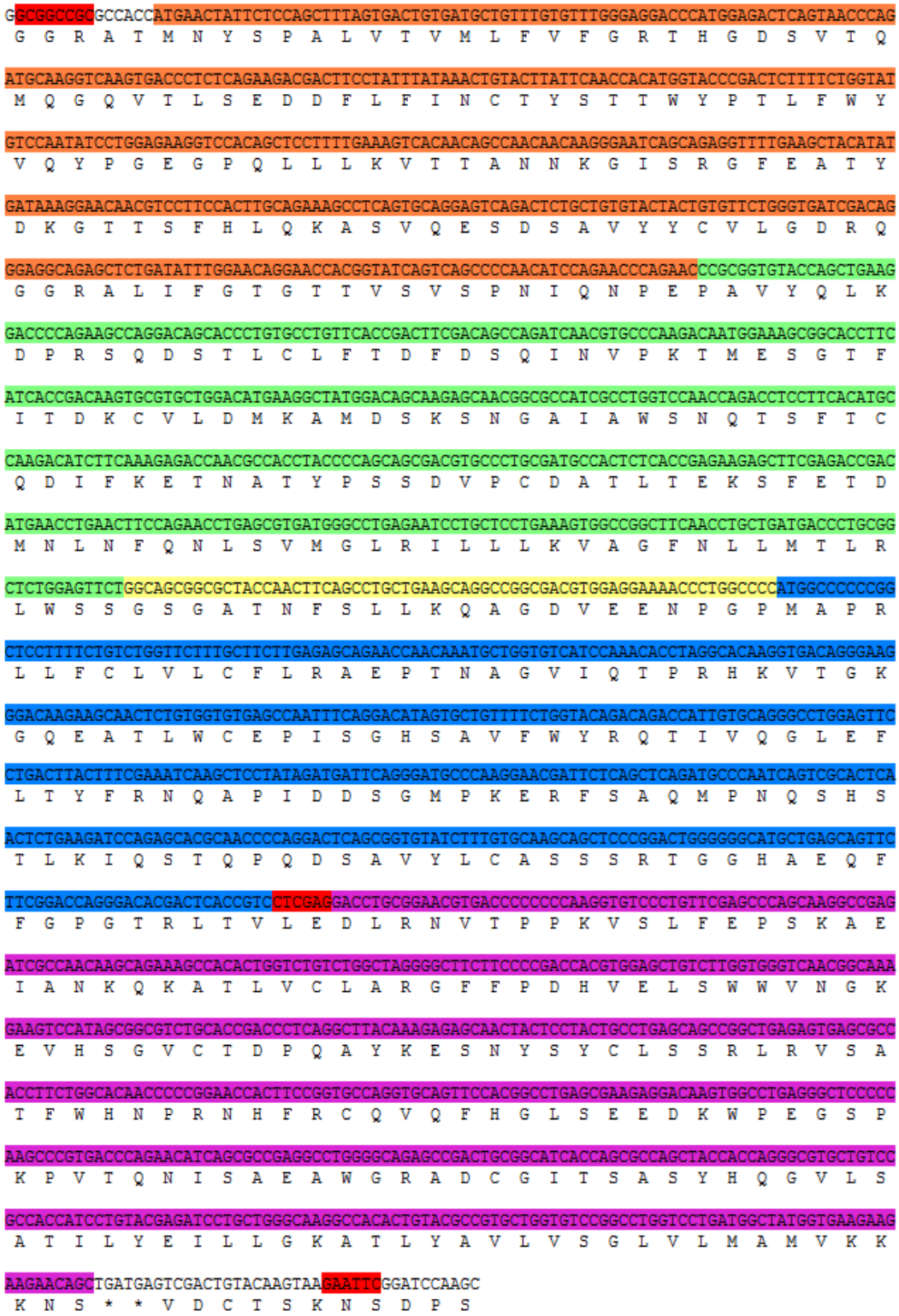
Zhang, L., Fung-Leung, W. & Miller, R.G., 1995. Down-regulation of CD8 on mature antigen-reactive T cells as a mechanism of peripheral tolerance. *Journal of immunology (Baltimore, Md. : 1950)*, 155(7), pp.3464–71.

Zhou, X. et al., 2003. Lentivirus-mediated gene transfer and expression in established human tumor antigen-specific cytotoxic T cells and primary unstimulated T cells. *Human gene therapy*, 14(11), pp.1089–105.

Zorn, E. et al., 2002. Infusion of CD4+ donor lymphocytes induces the expansion of CD8+ donor T cells with cytolytic activity directed against recipient hematopoietic cells. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 8(7), pp.2052–60.

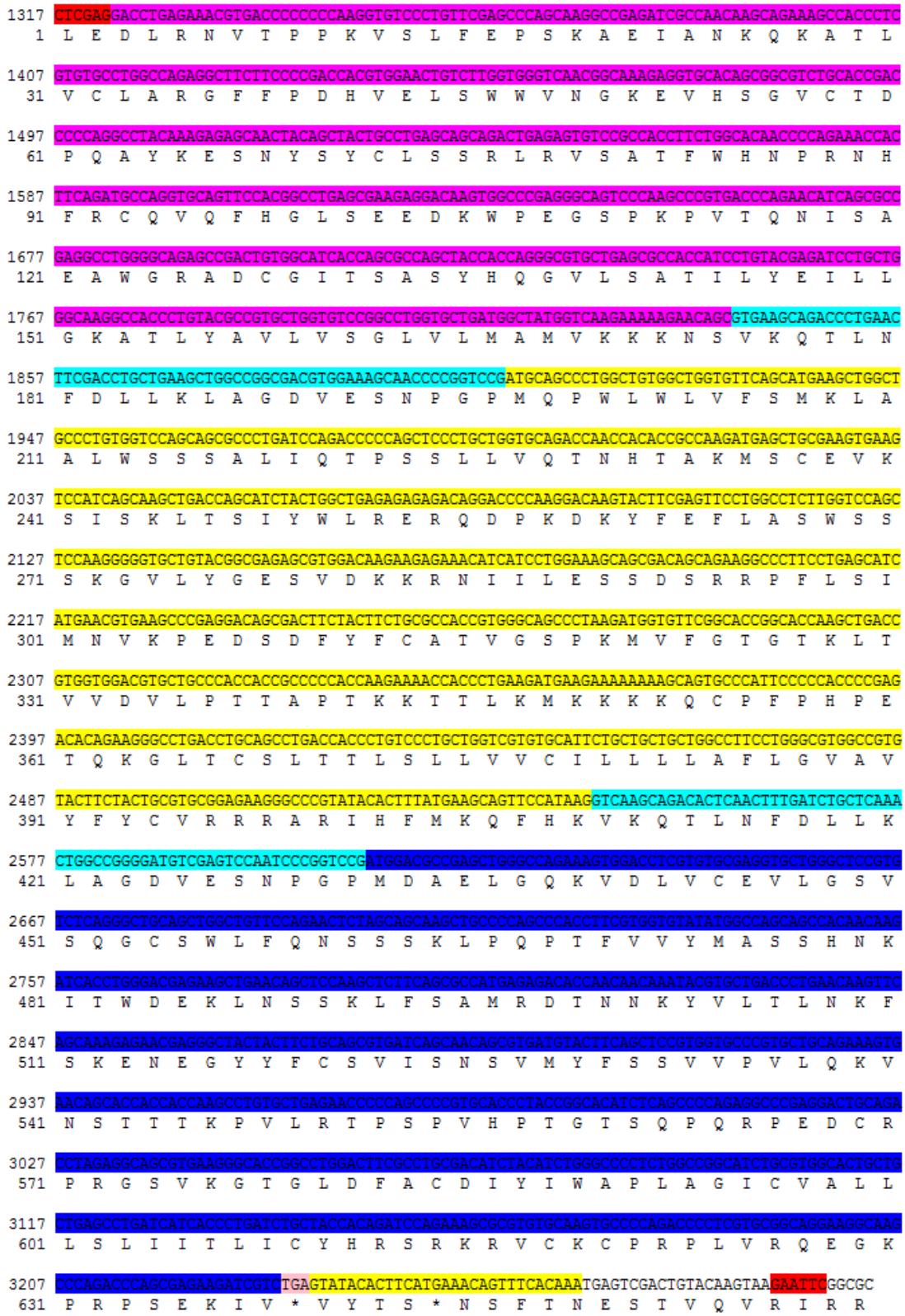
Appendices

I. Base sequence of F5-TCR used in F5-TCR pMP71 vector



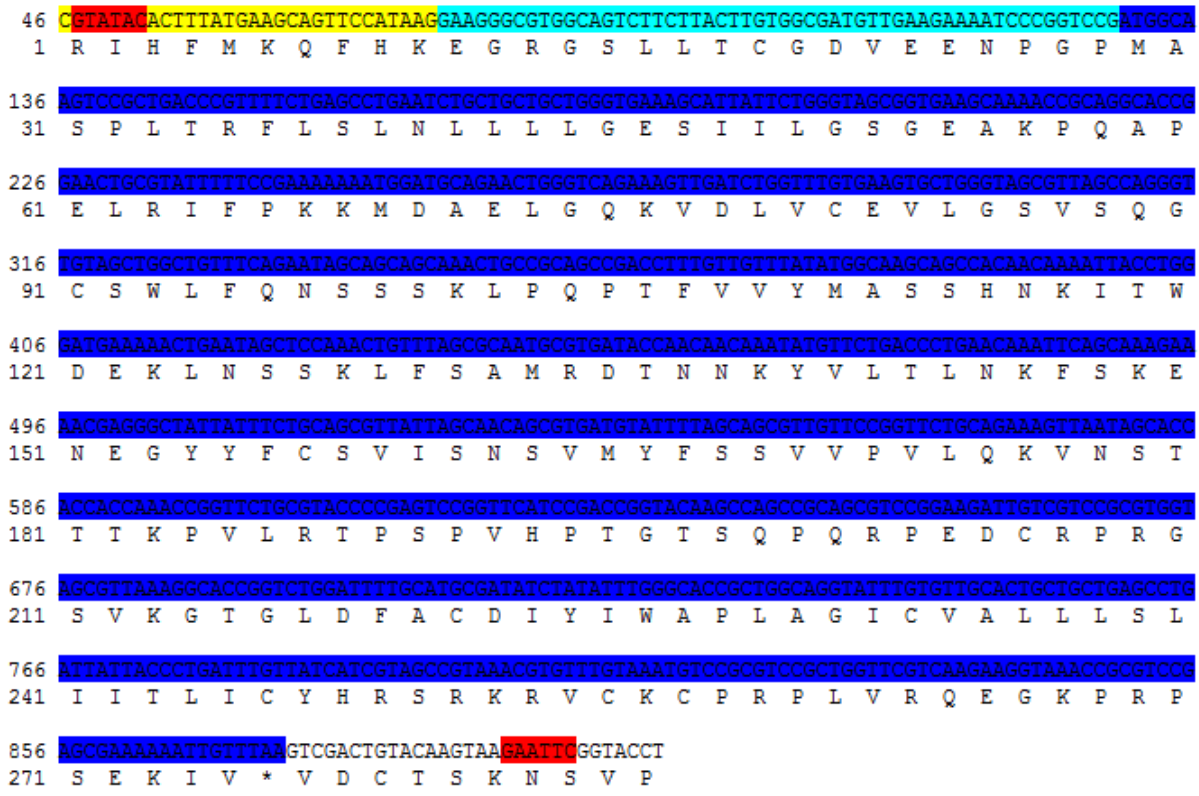
Appendix I Base sequence of F5-TCR used in the F5-TCR pMP71 vector. Restriction sites NotI, XhoI and EcoRI are shown in red. Transgenes include Vα (orange), Cα (green), P2A sequence (yellow), Vβ (blue), Cβ (purple).

II. Base sequence of Cβ-CD8αβ construct



Appendix II Base sequence of Cβ-CD8αβ construct made by Geneart.
Restriction sites XhoI and EcoRI are shown in red. Transgenes include Cβ (purple), F2A peptide (cyan), CD8β (yellow) and CD8α (blue).

III. Base sequence of revised CD8α construct



Appendix III Base sequence of revised CD8α construct made by Geneart.
Restriction sites BstZI and EcoRI are shown in red. Transgenes include 5’ portion of CD8β (yellow), T2A peptide (cyan) and CD8α (blue).